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HEMATOPOIETIC MECHANISMS*

Consulting Editor
WALTER S ROOT

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PREFACE

Walter S. Root

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This monograph deals with topics of considerable current interest Thus Dameshek[1] calls attention to the increasing use of the physiological approach in hematological studies made since 1925 Part I, devoted to techniques used in the quantitative study of blood cells, amply confirms his thesis

According to the available information, neither lack of oxygen nor neural factors constitutes the direct stimulus for red cell formation [2] This view has led investigators to studies of the hormone control of blood cell formation, the subject of Part II of this publication Destruction of blood cells by hemolytic mechanisms is also presented and well discussed in Part II

Of the many hormones that influence red blood cell formation, that postulated in 1906 by Cornot and Déflandre is currently the subject of intense investigation, and *hemopoietine* was the subject of Part III

Like other living organisms, red and white blood cells exist *for a finite time* Their life span has been measured in various ways, and the methods used, as well as the results obtained, are presented in Part IV

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Part I. Techniques Used in the Quantitative Study of Blood Cells

SOME QUANTITATIVE ASPECTS OF ERYTHROPOIESIS*

Clement A Finch

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Attention to numbers is most appropriate in examining the behavior of the erythron, since its abnormalities relate more to changes in the quantity of erythrocytes than to alterations in the function of gas transport by the individual cell The capacity of the marrow to produce red cells and regulatory r[illegible] in red cell production are topics discussed in this monograph. [illegible] studies that we have pursued relating to [illegible] erythron

The [illegible]

It would seem appropriate first to define the number of cells in the normal erythroid marrow This is equally pertinent to the consideration of leukopoiesis, since estimates of the myelocytic cells of the marrow are made by first determining the erythroid marrow cellularity and then converting this figure to that of the white cells through the erythroid-myeloid ratio of the marrow [1, 2] Several methods, each involving certain assumptions, have been employed to estimate the number of erythroid cells. Osgood[1] made calculations by assuming that one fourth of the nucleated red cells were capable of division and that the marrow maturation time of the other three fourths was forty-eight hours Knowing the circulating red cell turnover rate, he was able to estimate the number of red cell precursors. Patt[2] has made calculations based on the number of cells produced per hour (derived from the red cell life span), the number of mitoses among the nucleated red cells, and the time interval of mitosis We have employed a method that has been outlined in principle by Suit [3] This method depends on the enumeration of cells in a marrow aliquot and the relation of this aliquot to total marrow through a marrow tag (Fe^{59}) Radioiron was chosen for this purpose since it localizes temporarily in high concentration in the marrow normoblast In three animal species, two thirds of the extravascular radioiron at the time of minimum blood activity was found to be localized in erythropoietic tissues A similar distribution is assumed to apply in man The details of this method are published elsewhere [4] Results obtained in the rabbit and man are presented in TABLE 1 to permit comparison in two different species. For descriptive purposes the erythron has been divided into four classes of cells, (1) the nucleated red cell, (2) the marrow reticulocyte, (3) the circulating reticulocyte, and (4) the adult erythrocyte Of interest is the reticulocyte pool of the marrow, constituting approximately one half of the marrow erythroid

* The work described in this paper was supported by Contract AT-(45 1)-218 from the Atomic Energy Commission, Washington, D C, and by Research Grant H 2994 from the National Heart Institute, Public Health Service, Bethesda, Md

cells While quantitative estimates of the erythroid marrow in animal species have varied considerably, in normal man there has been surprising agreement. Thus, nucleated red cells have been determined by three different methods to be 3.4 to 8.6 × 10⁹ cells per kilogram [1-4, 6]

TABLE 1
CELLULAR COMPOSITION OF ERYTHRON

	Rabbit		Man	
	Cells/kg × 10⁹	Ratio	Cells/kg × 10⁹	Ratio
I Nucleated red cells	5.8	1.6	5.0	1.7
II Mature reticulocytes	4.3	1.2	4.7	1.5
III Circulating reticulocytes	11.6	3.1	3.1	1.0
IV Mature red cells	370	100	308	100

The fractional turnover of the cells in these 4 stages of development can be calculated from the above data and from the erythrocyte life span of 50 days in the rabbit[8] and 120 days in man. It is assumed that cell multiplication occurs only in the nucleated erythrocytes. Thus, the number of cells entering or leaving each of the 3 latter stages divided by the number of cells in each stage indicates the percentage of turnover per day. The results of these calculations are shown in TABLE 2

TABLE 2
TURNOVER OF ERYTHROID CELLS

	Fractional turnover (per cent per day)		Time for complete turnover (hours)	
	Rabbit	Man	Rabbit	Man
I Nucleated red cells	(120)	(51)	(20)	(47)
II Marrow reticulocytes	160	55	15	44
III Circulating reticulocytes	65	83	37	29
IV Mature red cells	2	0.8	1200	2880

A convenient method of estimating time spent in stages 1 and 2 is the measurement of the marrow transit time of radioiron. Radioiron injected intravenously disappears rapidly from the plasma and, shortly thereafter, reappears in circulating red cells. If the interval is taken between 50 per cent disappearance from the blood to 50 per cent release by the marrow, transit time is 24 hours in the rabbit and 82 hours in man (FIGURE 1). Since radioiron is taken up by the normoblast in the marrow throughout its development,[7] and even in small amounts by the reticulocyte,[8] this represents an

easily measured but somewhat arbitrary interval. Observations in patients with hematologic disorders show, in some instances, considerable shortening in transit time, particularly when anemia is severe in hemolytic disorders and when encroachment on marrow space occurs This shortening could be interpreted either as an acceleration of maturation time or as a premature release of developing red cells, our data to date support the latter interpreta-

change would appear to be that the normally required one- to two-day maturation of the reticulocyte in the marrow was now occurring in the circulating

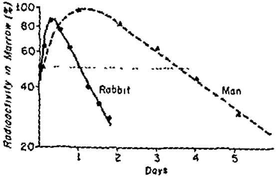


FIGURE 1 Marrow radioiron turnover. The values of marrow iron turnover are taken

release

blood Such a shift in the reticulocyte pool from the marrow to the blood has been demonstrated in phenylhydrazine anemia in rabbits[8] We have before us, then, a model of the numerical dimensions of the normal erythroid marrow and have characterized the rate of development of these cells. In two species, while the number of cells per kilogram is generally similar, appreciable differences are found in rates of cell production and maturation.

The Measurement of Erythropoietic Activity

There are available a number of clinical methods by which the activity of the erythroid marrow and the production and destruction of mature erythro-

be useful in the
ds directly reflect
red to as effective

erythropoiesis. Others reflect total marrow activity, whether or not it results in viable red cells (TABLE 3). Of obvious importance, if red cell survival measurements are to be used to calculate erythropoiesis, is the requisite that production and destruction be in equilibrium, or that the change in red cell mass be known There are other differences, such as the latent period in their response to changes in erythropoietic rate, which influence the usefulness of these measurements

TABLE 3
ERYTHROKINETIC MEASUREMENTS

	Production	Destruction
Effective erythropoiesis	Reticulocyte Red cell uptake of iron	Red cell life span
Total erythropoiesis	Erythroid/myeloid ratio Plasma iron turnover	Fecal urobilinogen

The study of more detailed methods of evaluating erythropoiesis has served only to emphasize the usefulness of the absolute reticulocyte count as an indicator of effective red cell production To be sure, there are circumstances that alter the linear relationship between the number of reticulocytes and the number of red cells produced, these relate particularly to the duration of the circulating reticulocyte stage [5, 11] In megaloblastic anemias, many cells lose reticulum before entering circulation [12] More frequent, however, is the premature release of the marrow cell [9] This shortens the time spent in the marrow, but correspondingly lengthens the reticulocyte stage in circulation This shift may be recognized by the presence of "young" reticulocytes with heavy reticulum and nucleated red cells Since marrow reticulocytes are slightly more numerous than circulating reticulocytes, the reticulocyte count may be doubled by the early release of marrow reticulocytes into the blood This mechanism probably accounts for much of the variation in maturation time of reticulocytes in the circulating blood, although other factors are of importance and bear further investigation Proper allowance for the reticulocyte shift makes the reticulocyte count, when expressed in absolute numbers rather than percentages, a valuable index of erythropoiesis

Erythropoietic Response to Anemia

Since there is no exact yardstick by which erythropoietic activity can be measured we have ...

te

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easily measured but somewhat arbitrary interval. Observations in patients with hematologic disorders show, in some instances, considerable shortening in transit time, particularly when anemia is severe in hemolytic disorders and when encroachment on marrow space occurs. This shortening could be interpreted either as an acceleration of maturation time or as a premature release of developing red cells, our data to date support the latter interpretation For example, among patients with hemolytic anemia with and without shortening of marrow radioiron transit time there was no discernible difference *in the rate of new cell output per nucleated marrow cell* The only change would appear to be that the normally required one- to two-day maturation of the reticulocyte in the marrow was now occurring in the circulating

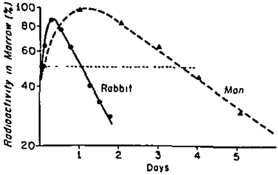


FIGURE 1 Marrow radioiron turnover The values of marrow iron turnover are taken

release

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The Measurement of Erythropoietic Activity

... number of clinical methods by which the activity of

the production or destruction of circulating red cells, ...

marrow activity are greatly elevated, those reflecting effective erythropoiesis are normal or depressed. In some of these conditions the words "maturation arrest" have been applied It seems more likely, in view of the turnover of pyrolle pigment and iron and the frequency of mitosis in these marrows, that the erythroid precursors are being destroyed either in the marrow or after an extremely brief sojourn of at most a few hours in the blood The term

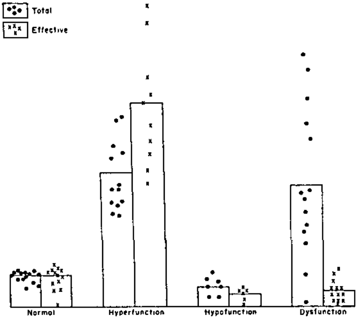


FIGURE 2 Comparison of total and effective erythropoiesis as measured by plasma iron turnover versus reticulocyte count These data are taken from a previous article and illustrate measurements of total and effective erythropoiesis in thirty-four patients with anemia

"dyspoietic anemia" would seem appropriate when the greater portion of erythropoietic activity is ineffective in the production of viable erythrocytes

Summary

This paper has been a summary largely of the observations made by a number of individuals in our laboratory during the past few years Its purpose has been to indicate some of the methods employed and results obtained, relating particularly to the quantitation of erythropoiesis in man It is not so much a question of whether erythropoiesis can be quantitated, but rather how exact this quantitation may be in a given situation It is also evident that while one may establish patterns of marrow response, there is as yet but a primitive and superficial concept of the processes of cell construction

The classification of erythropoiesis in anemic man, based on such measurements, may be simply stated to include: (1) normal marrow hyperfunction, (2) relative or absolute marrow hypofunction, and (3) dysfunction. The range of normal hyperfunction has been most accurately defined in acute a[illegible] induced by phlebotomy, where iron stores were sufficient to permit [illegible] In such cases erythropoiesis could be quanti[illegible] [illegible]nt of blood removed. Rates of erythropoiesis [illegible] [illegible]re observed.[15] Studies in patients with chronic hemolytic anemia [illegible] hat the maximum capacity of erythropoiesis may be at a level of six to eight times normal[10, 11], recently Crosby[16] provided evidence that bleeding done during a period of months

TABLE 4

	Effective*		Total*	
	Reticulocyte count	Red cell uptake of radioiron	Erythroid marrow	Plasma iron turnover
Hyperfunction				
Hereditary spherocytosis	6 8	3 6	6 4	5 3
Hereditary spherocytosis	8 8	4 2	4 7	5 3
Relative hypofunction				
Myelofibrosis	0 8	1 3	—	1 9
Cirrhosis	0 9	1 3	2 0	1 1
Hypofunction				
Uremia	0 2	0 3	0 2	0 6
Aplastic anemia	0 2	0 1	0 1	0 5
Dysfunction				
Cooley's anemia	0 7	0 6	11	6 5
Pernicious anemia	—	1 2	—	2 8

* Values expressing effective and total erythropoiesis are expressed in relation to normal erythropoiesis

results in an increa[illegible] [illegible]te of three times normal to this higher level [illegible] anemia illustrating maxim[illegible] function is defined as a subnormal produc[illegible] of patients with uremia and aplastic anemia in TABLE 4) Relati[illegible] hypofunction implies erythroid activity at a normal or increased level, but significantly less than that expected of the normal marrow subjected to the stimulus of anemia. Illustrative cases with myelofibrosis and cirrhosis are also shown in TABLE 4. In these three degrees of marrow response there is reasonable agreement between the various measurements of effective and total erythropoiesis.

Marked discrepancies in measurements occur in certain anemias such as Cooley's anemia,[18] pernicious anemia,[12] and other anemias with hyperplastic erythroid marrow, but reticulocytopenia. Measurements reflecting total

THE PRODUCTION AND DESTRUCTION OF GRANULOCYTES IN NORMAL AND LEUKEMIC MAN*

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In the normal hematopoietic state, the rate of production of leukocytes is equal to the rate of their destruction. The destruction of a leukocyte is related inversely to its life span, that is, the more rapid the rate of destruction, the shorter the life span of the cell. The rate of production of leukocytes may be determined if the rate of destruction is known.

The life span of a leukocyte is the period of time from the initial differentiation of the primitive blood cell until its death. An indeterminable number of reproductive mitoses may occur in this period, resulting in a variable life span. Once the leukocyte is no longer able to divide (postmitotic), the remaining period of survival is fixed and predictable. An estimate of the rate of destruction of leukocytes may be derived from this fixed value. A portion of the life of the postmitotic granulocyte (PMN) is spent in the circulating blood. Another portion of this period is spent in the readily available leukocyte reservoir and in the hematopoietic site prior to delivery into this reservoir and into the circulating blood.[1] The life span of the postmitotic granulocyte was termed the intravascular residence time (IRT).

The ultimate goal of normal granulocytopoiesis is the development of mature functioning granulocytes, that is, the polymorphonuclear leukocytes (PMNs). Therefore PMN production represents effective leukopoiesis as contrasted with the production of immature granulocytes, that is, myelocytes that for one reason or another may be unable to mature. Many investigators have, with variable results,[2-17] studied the life span of leukocytes both in animals and in man (TABLES 1, 2, and 3). A study of the rates of production and destruction of the PMN should offer a valid comparison of effective leukopoiesis in the normal subject and in patients with granulocytic leukemia.[18]

Patients and Methods

These data were obtained from repeated studies on 23 nonleukemic patients and 10 patients with granulocytic leukemia.

Standard hematologic methods for total leukocyte and differential counts with NBS-certified pipettes and hemocytometers were employed. The leukocyte counts were performed with an accuracy of ± 8.8 per cent within 70 per cent confidence limits. The blood volume was calculated as 8 per cent of the body weight.

* The work reported in this paper was supported in part by Research Grant C 2272 from the National Cancer Institute, Public Health Service, Bethesda, Md., the Leukemia Research Foundation, Inc., Los Angeles, Calif., and the Lakow Foundation, El Segundo, Calif.

and maturation involved in erythropoiesis The quantitative evaluation of marrow function, however, deserves a place together with etiological and morphologic considerations in the clinical diagnosis and characterization of anemia

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. . . determined by three methods (1)
. . . pheresis

Time of Disappearance of . . .

In 1930 Weiskotten[4] administered benzol to rabbits in doses that effectively halted production of granulocytes The disappearance of the neutrophil (amphophil) from the peripheral blood led to the conclusion that the survival time or intravascular residence time of the rabbit PMN was about . . . IRT of the PMN was determined *in man* by this method, fol- . . . depressant agent dimethyl Myleran, . . . mixed population of PMNs was found[20] IRT values . . . grouped arbitrarily as IRT_1, from 6 0 to 11 9 days were termed IRT_2, and 12 0 to 17 9 days, IRT_3 Values of 18 0 days and longer were grouped as IRT_4

In the nonleukemic subject, the PMN reservoir was estimated to be at least 60 times the circulating PMN number[21] Therefore, the rates of PMN production in the nonleukemic subjects are minimal rates, subject to appropriate increases if and when the size of the total PMN reservoir is proved to be larger

In patients with granulocytic leukemia with granulocyte concentrations . . . the extracirculatory reservoir was calculated to . . . locytes For patients . . . trations below 20,000/cu mm, . . . e 60 times the total granulocyte number in the circulation . . . of granulocyte concentration from 20,000 to 63,000/cu mm, there are no data available for determining the size of the reservoir

Leukopheresis

The rate of PMN delivery was estimated from direct measurement of the number of PMNs replenishing the depleted reservoirs during and immediately following leukopheresis From 3 8 to 160 × 10^9 PMNs were removed during time intervals ranging from 34 to 256 min in 47 studies in 23 patients with neoplastic diseases other than leukemia The total number of PMNs necessary to replace those removed, divided by the time required to regain the initial PMN concentration, gives an approximation of production and delivery of PMNs for this period Ten patients *with granulocytic* leukemia were studied on 14 occasions

TABLE 1
AVERAGE SURVIVAL TIME OF THE POSTMITOTIC LEUKOCYTE IN ANIMALS

Animal	Leukocyte type	Average survival time	Investigator
Rat	Granulocyte	23 min	VanDyke & Huff, 1951[3]
Rat	Lymphocyte	170 min	VanDyke & Huff, 1951[3]
Rabbit	Neutrophil	3–4 days	Weiskotten, 1930[4]
Rabbit	WBC	30 min	Weisberger *et al.*, 1950[5]
Rabbit	WBC	3 days	Farr, 1951[6]
Rabbit	Lymphocyte	288 min	Yoffey, 1950[7]
Cat	WBC	16–24 hours	Lawrence *et al.*, 1945[8]

TABLE 2
AVERAGE SURVIVAL TIME OF THE POSTMITOTIC LEUKOCYTE IN NORMAL MAN

Leukocyte type	Average survival time	*Investigator*
Granulocyte	4 days	Roberts & Kracke, 1930[9]
Granulocyte	9 days	Ottesen, 1954[10]
Granulocyte	9 days	Hamilton, 1957[11]
WBC	13 days	Kline & Clifton, 1952[12]
WBC	30–90 min	White, 1954[13]
WBC	16 days, 50%	Weisberger & Levine, 1954[14] Weisberger & Suhrland, 1955[15]
Lymphocyte	3–4 and 100–200 days	Ottesen, 1954[10]
Lymphocyte	3–4 and 100–200 days	Hamilton, 1957[11]

TABLE 3
AVERAGE SURVIVAL TIME OF THE POSTMITOTIC LEUKOCYTE IN MAN WITH THE LEUKEMIAS

Type of Leukemia	Average survival time	Investigator
Granulocytic	3 days	Osgood *et al.*, 1952[16]
Lymphocytic	7 days	Osgood *et al.*, 1952[16]
Acute	9–11 days, 50%	Weisberger & Levine, 1954[14] Weisberger & Suhrland, 1955[15]
Granulocytic	5 days	Osgood *et al.*, 1954[17]
Granulocytic	15–16 days, 50%	Weisberger & Levine, 1954[14] Weisberger & Suhrland, 1955[15]
Granulocytic	23 days	Hamilton, 1957[11]
Lymphocytic	30 days	Osgood *et al.*, 1952[16]
Lymphocytic	84 days	Osgood *et al.*, 1954[17]
Lymphocytic	85 & 300 days	Hamilton, 1957[11]

of PMNs produced In the normal individual a greater portion of newly formed cells will appear in the reservoirs, which contain 60 times the number of cells present in the peripheral blood

Results

Destruction of PMNs

Patients without hematologic abnormality The intravascular residence time of the PMNs was determined in 7 patients following the administration of hematodepressant doses (0 5 to 0 9 mg /kg body weight) of CB-2348 and CB-2348K Two populations of PMNs were measured with mean intravascular residence times of 8 6 ± 1 7 days and 13 6 ± 2 0 days (TABLE 4)

Patients with granulocytic leukemia Similar observations on the IRT of PMNs were made on 10 patients with granulocytic leukemia on 19 occasions following the administration of CB-2348 and CB-2348K in therapeutic doses of 0 4 to 0 8 mg /kg body weight (TABLE 4) In 5 patients with chronic granulocytic leukemia 2 PMN populations were found, the mean IRT_2 was 8 4 ± 1 6 days, and the IRT_4 was 23 7 ± 3 7 days These values differed from those of normal subjects by the presence of a long-lived IRT_4 and the absence of an IRT_3

In 3 patients with chronic granulocytic leukemia who had progressed toward an acute phase, 4 populations of PMNs were found with a mean IRT_1 of 4 9 ± 0 4 days, an IRT_2 of 8 9 ± 2 0 days, an IRT_3 of 15 2 ± 1 6 days, and an IRT_4 of 18 6 ± 0 9 days The appearance of a short-lived IRT_1, found neither in normal subjects nor in patients with chronic leukemia, is of interest The difference between the IRT_4 of 18 6 days from that of the IRT_3 of 15 2 days was significant to less than the 0 05 level

In 2 patients with apparent primary acute granulocytic leukemia the mean IRT_1 was 3 7 ± 1 06 days, IRT_2 was 7 8 ± 0 5 days, and IRT_3 was 13 6 days An IRT_4 was not found

Production of PMNs

Computed from the IRT in patients without hematological abnormality The minimal rate of PMN production was computed by dividing the IRT into the total number of PMNs in the peripheral blood and extracirculatory reservoirs The rates of PMN production in 14 patients on 19 occasions

TABLE 4
CB-2348 AND K (IV) IN 7 NONLEUKEMIC PATIENTS

Patient	IRT_1	IRT_2	IRT_3	IRT_4
GUA	—	9 7	17 1	—
SHE	—	—	11 5	—
SHA	—	6 4	12 8	—
BRU	—	8 7	14 7	—
BEN	—	9 5	13 8	—
ARO	—	10 6	14 0	—
STU	—	6 8	11 5	—
$\bar{x}$	—	8 62	13 63	—
SD	—	±1 71	±1 96	—

IRT VALUES IN 10 PATIENTS WITH GRANULOCYTIC LEUKEMIA IN 19 STUDIES

Patient	Chronic Granulocytic Leukemia			
	IRT_1	IRT_2	IRT_3	IRT_4
BIC	—	9 4	—	—
GOL 1	—	—	—	21 1
GOL-2	—	—	—	20 6
LEE	—	6 4	—	27 7
LUS	—	9 4	—	29 4
RAT-1	—	10 0	—	20 8 25 4
RAT-2	—	6 9	—	21 1
$\bar{x}$	—	8 4	—	23 7
SD	—	±1 6	—	±3 7
	Chronic Granulocytic Leukemia to Acute			
BUR-1	—	7 7 11 5	—	—
BUR-2	4 3	11 4	—	—
BUR-3	5 0	6 6	—	19 2
FAL-1	—	—	14 3	—
FAL-2	—	6 8	17 2	—
FAL-4	5 2	10 0	—	18 0
ROB-1	4 9	10 8	—	—
ROB-2	—	7 4	13 6	—
ROB 3	—	7 7	15 6	—
$\bar{x}$	4 9	8 9	15 2	18 6
SD	±0 4	±2 0	±1 6	±0 9
	Acute Granulocytic Leukemia			
WIL	2 9	7 4	—	—
LUT-1	—	8 1	13 6	—
LUT-2	4 4	—	—	—
$\bar{x}$	3 7	7 8	13 6	—
SD	±1 06	±0 5	—	—

TABLE 6

PRODUCTION OF PMNs COMPUTED FROM THE IRT OF THE PMN IN 10 PATIENTS WITH GRANULOCYTIC LEUKEMIA

Patients	Initial WBC No $\times 10^3$	Initial PMN No $\times 10^3$	BV $\times 10^2$	Total PMNs in PB $\times 10^{11}$	Total PMNs in body $\times 10^{11}$	IRT_1	Minimal daily prod IRT_1 $\times 10^9$	IRT_2	Minimal daily prod IRT_2 $\times 10^9$	IRT_3	Minimal daily prod IRT_3 $\times 10^9$	IRT_4	Minimal daily prod IRT_4 $\times 10^9$	Body wt kg	Prod /kg /day IRT_1 $\times 10^9$	Prod /kg /day IRT_2 $\times 10^9$	Prod /kg /day IRT_3 $\times 10^9$	Prod /kg /day IRT_4 $\times 10^9$
Chronic Granulocytic Leukemia																		
BIC	246 0	185 0	5 5	9 83	18 66	—	—	9 4	198 6	—	—	—	—	66 0	—	3 00	—	—
GOL-1	325 0	157 0	6 7	10 18	20 56	—	—	—	—	—	—	21 1	96 4	84 0	—	—	—	1 35
GOL-2	215 0	128 0	6 7	8 55	17 16	—	—	—	—	—	—	20 6	81 3	84 0	—	—	—	1 00
LEE	495 0	299 0	4 0	11 95	21 90	—	—	6 4	373 0	—	—	22 2	86 3	50 0	—	7 48	—	1 72
LUS	12 6	12 6	6 0	0 26	41 60	—	—	9 4	485 0	—	—	29 4	155 0	25 0	—	6 47	—	2 06
RAT 1	422 0	251 0	4 6	11 62	23 24	—	—	10 0	212 4	—	—	20 8	112 0	57 5	—	4 04	—	1 93
												25 4	91 5					1 59
RAT 2	310 0	210 0	4 6	9 65	19 34	—	—	6 9	282 0	—	—	21 1	91 7	57 5	—	4 87	—	1 59
Chronic Granulocytic Leukemia → Acute Granulocytic Leukemia																		
								7 7	32 5							0 50		
BUR 1	64 0	24 0	5 2	1 25	2 50		—	11 5	21 7	—	—	—	—	65 0	—	0 33	—	—
BUR 2	69 0	27 6	4 8	1 32	2 64	4 3	61 4	11 4	23 2	—	—	—	—	60 0	1 02	0 39	—	—
BUR 3	19 1	9 2	4 8	0 52	31 20	5 0	624 0	6 6	473 0	—	—	19 2	163 0	60 0	10 40	7 88	—	2 71
PAL-1	64 7	38 0	3 2	1 22	2 44	—	—	—	—	14 1	17 1	—	—	40 0	—	—	0 43	—
PAL-2	65 8	21 8	3 2	0 70	1 40	—	—	8 8	20 6	17 2	8 2	—	—	40 0	—	0 51	0 21	—
PAL-4	87 6	18 8	3 2	0 60	1 20	5 2	23 1	10 0	12 0	—	—	18 0	6 7	40 0	0 43	0 30	—	0 17
ROB 1	24 7	15 0	4 6	0 69	1 38	4 9	28 1	10 8	12 8	—	—	—	—	37 5	0 49	0 22	—	—
ROB 2	111 5	19 0	4 6	0 88	1 76	—	—	7 4	23 3	13 6	12 9	—	—	57 5	—	0 41	0 22	—
ROB 3	67 4	3 4	4 3	0 15	0 30	—	—	5 7	4 0	15 6	3 92	—	—	54 0	—	0 072	0 036	—
Acute Granulocytic Leukemia																		
WIL	6 0	2 5	6 0	0 15	4 00	2 9	310 0	7 4	121 5	—	—	—	—	25 0	4 14	1 63	—	—
LUT 1	708 0	55 0	4 3	2 50	5 00	—	—	8 1	61 8	13 6	36 8	—	—	54 0	—	1 14	0 68	—
LUT 2	229 2	4 2	3 8	0 16	0 32	4 4	7 1	—	—	—	—	—	—	47 5	0 155	—	—	—

TABLE 5
RATE OF PRODUCTION OF PMNs FOLLOWING HN2, CB-2348, AND CB-2348K

Patient	$\times 10^3$ Initial PMN	$\times 10^3$ BV	$\times 10^9$ Total PB PMN	$\times 10^9$ Total body PMN	Days IRT_1	$\times 10^9$ Minimal daily prod 1	Days IRT_2	$\times 10^9$ Minimal daily prod 2	Days IRT_3	$\times 10^9$ Minimal daily prod 3	Body wt kg	PMN Prod IRT_1 kg/day $\times 10^9$	PMN Prod IRT_2 kg/day $\times 10^9$	PMN Prod IRT_3 kg/day $\times 10^9$
MLR-1	3 30	1 1	3 63	218	—	—	7 9	27 6	12 1	18 00	14 3	—	1 92	1 26
MLR-2	2 80	1 1	3 08	185	3 4	54 5	9 2	19 8	—		13 6	4 03	1 44	—
WIT	5 30	1 1	5 83	350	4 5	78 0	8 2	42 6	—		10 4	7 80	4 08	—
JEN 2	7 10	1 3	9 23	555	4 3	129 0	—	—	—	—	16 0	8 05	—	—
JEN-3	3 30	1 3	4 30	258	5 2	49 8	—	—	—	—	16 0	3 12	—	—
CAS-2	7 30	1 5	10 95	657	—	—	6 6	99 5	—	—	18 6	—	5 34	—
CAS-3	4 10	1 5	6 15	370	5 2	70 8	—	—	—	—	18 6	3 78	—	—
COP	3 30	3 4	11 20	672	—	—	6 3	107 0	—	—	42 7	—	2 52	—
CAR	5 65	3 9	22 00	1320	3 9	338 0	6 6	202 0	—	—	49 0	6 90	4 08	—
ROS	5 90	4 3	25 40	1520	—	—	7 3	209 0	—	—	54 0	—	3 86	—
PRE-1	5 50	4 6	25 30	1518	—	—	10 4	146 0	—	—	58 0	—	2 52	—
PRE-2	6 55	4 6	30 20	1810	—	—	8 4	216 0	—	—	58 0	—	3 72	—
CLA	8 10	3 6	29 20	1750	3 2	548 0	6 7	262 0	—	—	45 0	12 20	5 82	—
ILE	9 40	4 8	45 00	2700	4 2	645 0	7 7	351 0	—	—	60 0	10 80	5 88	—
CRO	4 10	5 6	23 00	1380	—	—	7 6	182 0	—	—	70 0	—	2 58	—
KRA	5 80	6 0	34 80	2090	—	—	9 9	211 0	—	—	76 0	—	2 76	—
PRI	8 00	6 5	52 00	3120	5 4	577 0	—	—	13 5	231 00	80 9	7 15	—	2 88
GRU-1	2 90	5 8	16 80	1010	—	—	9 6	105 0	13 2	76 21	72 0	—	1 44	1 08
GRU-2	4 20	5 8	24 40	1460	5 5	266 0	—	—	—	—	72 0	3 72	—	—
CAS 4	15 80	1 7	26 80	1610	3 2	503 0	6 3	255 0	—	—	21 0	23 90	12 12	—
FEN	29 00	3 5	101 50	6100	3 8	1600 0	—	—	—	—	44 0	36 40	—	—
Total	147 40	—	510 77	30,653	51 8	4859 1	118 7	2435 5	38 8	323 21	—	127 65	60 08	
$\bar{x}$	7 01	—	24 32	1459 67	4 3	404 9	7 9	162 37	12 9	107 74	—	10 63	4 01	
No	21 00	—	21 00	21.0	12 0	12 0	15 0	15 0	3 0	3 0	—	12 00	15 00	

ROS	8/06/57	50	36.5	80	2.9	5.0	4.8	200	182.5	262.8	4.86	54
KOT	8/12/55	18	14.0	96	3.5	5.6	3.8	50	140.0	201.6	3.66	55
MAA	5/01/56	26	23.4	105								55
PRE	7/13/56	25	15.6	58	4.5	3.5	4.5	120	130.0	187.2	3.22	58
PRE	12/11/56	52	41.0	246	6.0	5.5	6.0	276	148.5	213.8	3.68	58
PRE	12/20/56	84	69.7	242	6.0	3.4	6.0	300	232.3	334.5	5.76	58
FLE	3/05/56	88	83.0	141	18.0	21.0		360	230.5	331.9	5.53	60
TAL	12/08/55	43	38.0	53	11.85	14.4		175	217.1	312.6	5.21	60
STU	4/16/57	81	68.2	184	7.5	5.5	7.2	290	235.1	338.5	5.45	62
CRO	7/30/57	100	75.8	147	4.0	4.3	4.0	160	473.7	682.1	9.74	70
CRO	8/02/57	108	82.0	177	3.8	2.7	3.8	275	298.1	429.3	6.13	70
CRO	8/09/57	87	62.9	106	5.5	10.4	5.5	275	232.0	334.0	4.77	70
PAS	7/19/56	8	6.2	71	3.2	3.9		Insufficient data				72
PAS	7/20/56	5	3.8	64	3.4	3.0		Insufficient data				72
PAS	7/25/56	9	7.7	126	4.0	4.8		Insufficient data				72
PAS	7/27/56	17	13.4	97	4.0	3.3		Insufficient data				72
KRA	6/21/57	44	36.1	187	4.9	5.0	5.0	187	193.0	277.9	3.70	75
KRA	6/29/57	53	39.6	243								75
KRA	7/06/57	43	33.3	157	4.9	5.3	5.3	157	212.0	305.3	4.07	75
MCC	7/16/57	8	5.6	34	3.8	5.3	5.3	<34	<164.7	<237.2	<3.00	79
PRI	2/28/56	73	65.0	176	4.9	11.3	4.9	465	139.7	201.2	2.43	81
PRI	3/02/56	33	28.0	111	8.6	8.1	8.1	240	116.6	167.9	2.07	81
GRU	10/29/57	51	36.4	210	3.4	4.2	3.7	232	156.8	225.8	3.13	72
GRU	11/05/57	60	47.0	189	5.0	3.4	5.0	405	116.0	167.0	2.31	72
GRU	11/15/57	61	47.2	224	5.7	3.9	4.6	400	118.0	169.9	2.35	72
HAZ	1/17/58	10	8.4	78	2.3	2.6	2.6	78	107.6	154.9	2.42	64

TABLE 7
PRODUCTION FOLLOWING LEUKOPHERESIS

Patient	Date	Total WBC removed $\times 10^9$	Total PMN removed $\times 10^9$	Time leukopheresis min	Initial PMN count $\times 10^3$	End PMN count $\times 10^3$	Return to initial count $\times 10^3$	Time of return min	Rate of replenishment /min $\times 10^6$	Rate of replenishment /day $\times 10^9$	Rate of replenishment /kg /day $\times 10^9$	Kg
MER	12/20/55	14	11.5	56	4.0	6.8	4.5	160	71.8	103.4	9.94	10.4
MER	12/29/55	12	8.5	46	3.3	4.1	3.3	80	106.2	152.9	14.70	10.4
MER	12/30/55	13	9.1	63	2.9	4.3	2.9	247	36.8	52.9	5.1	10.4
MER	1/24/56	16	13.4	232	3.4	3.6	3.6	232	57.7	83.1	7.99	10.4
WIT	7/02/57	19	13.9	146	3.3	1.5	2.8	387	35.9	51.7	4.97	10.4
CAS	3/20/56	23	17.5	256	8.4	5.5	7.5	330	53.0	76.3	4.02	19
CAS	3/21/56	13	9.2	68	5.5	6.1	5.5	118	135.2	194.7	10.24	19
CAS	7/03/56	54	44.0	124								19
CAS	1/08/57	45	33.2	127	6.0	3.0	6.0	215	154.4	222.3	10.58	21
CAS	1/15/57	21	17.4	88	5.0	5.35	5.35	88	197.0	283.7	13.50	21
JEN	5/07/56	37	26.0	205	5.3	5.3	5.3	205	126.8	182.6	11.41	16
JEN	6/19/56	25	19.0	158	5.0	2.5	5.0	300	63.3	91.2	5.70	16
JEN	6/26/56	21	18.5	147	7.0	4.6	7.0	300	61.6	88.7	5.54	16
COP	6/08/56	34	28.4	163	5.5	5.5	5.5	163	174.4	251.3	5.98	42
FEN	12/27/56	172	160.1	155	33.5	28.6	28.6	155	103.2	148.6	3.37	44
CLA	2/21/56	42	35.7	223	8.8	8.3	8.3	223	160.0	230.4	5.12	45
CAR	3/12/57	82	63.1	213	5.2	7.5	7.5	<213	<296.2	<426.5	<8.70	49
CAR	3/19/57	106	83.8	161	6.6	10.2	6.8	285	294.0	423.3	8.63	49
ORA	3/26/57	26	23.4	75	8.0	6.5	8.0	280	83.5	120.2	2.40	50
ORA	4/02/57	44	38.7	83	6.4	7.2	6.4	75	516.0	743.0	14.90	50
ORA	4/09/57	23	20.4	80	5.5	6.3	5.5	200	102.0	146.9	2.93	50

for the 3 chronic granulocytic leukemic patients in relapse, and 0.68 to 4.14 × 10^9 PMNs/kg/day for the 2 acute granulocytic leukemic patients.

Measurements from leukopheresis in patients without hematological abnormality. The rate of replenishment of PMNs was determined following continuous leukocyte withdrawal in 23 patients on 47 occasions. The return of PMNs to the initial level occurred within 6 hours following the end of leukopheresis. The rates of replenishment (production plus delivery) were measured at 2.07 to 14.90 × 10^9 PMNs/kg body weight/day (TABLE 7). Comparable data in patients with granulocytic leukemia are not available at this time.

Recovery from leukopenia in patients without hematological abnormality. The rates of production of PMNs measured in 10 patients on 11 occasions by this method varied from 0.86 to 5.54 × 10^9 PMNs/kg/day (TABLE 8).

Recovery from leukopenia in patients with granulocytic leukemia. Eight patients were studied for rates of PMN replenishment of reservoirs and blood following severe hematodepressant doses of CB-2348 or CB-2348K (TABLE 8). The rates of PMN production estimated by this method varied from 0.19 to 11.1 × 10^9 PMNs/kg/day.

Discussion

The rates of PMN production estimated from three different methods are within the same order of magnitude for the patients without hematological abnormality (TABLE 9). The data obtained from patients with granulocytic

TABLE 9
COMPARISON OF RATES OF PMN PRODUCTION IN MAN × 10^9/KG BW/24 HOURS

Method	Minimum	Maximum	
Repopulation	0.86	5.54	Recovering marrow
IRT	1.08	12.20	Minimal rate (RAR* = 60 × PB)
Leukopheresis	2.07	14.90	Replenishment <6 hours

* Readily available reservoir

leukemia, while not as extensive as those obtained in the nonleukemic subjects, nevertheless indicate that the leukemic state is not always characterized by an excessive proliferation of leukocytes. Indeed, leukopoiesis in granulocytic leukemia may be above, within, or below the normal range.

The intravascular residence times of the PMNs found in these patients with or without granulocytic leukemia are within the estimates reported by other investigators (TABLES 2 and 3).

The long-lived (IRT_1) population of the PMNs in chronic granulocytic leukemia appears to be characteristic.[11, 14] The data indicate that, as the conversion toward an acute granulocytic phase progressed, there was a shift from the long-lived PMN populations toward the short-lived populations. An increase in rate of production of immature granulocytes may occur during

TABLE 8

PRODUCTION RATE OF POLYMORPHONUCLEAR LEUKOCYTES PER KILOGRAM BODY WEIGHT FOLLOWING CB-2348, CB-2348K OR HN2

Patient	Diagnosis	BV in liters	Nadir $\times 10^3$	Peak $\times 10^3$	Peak minus nadir $\times 10^3$	Days	Total PMNs $\times 10^9$ returned to PB	Total PMNs $\times 10^9$ in PB and reservoirs	PMN/day $\times 10^9$	Weight kg	Total daily prod $\times 10^9$ kg
				Patients Without Hematological Abnormality							
ORA	Carcinoma of lung	4.0	2.5	4.3	1.8	10	7.20	432.0	43.2	50.0	0.86
CAS	Ewing's sarcoma	1.5	0.15	3.9	3.75	18	5.60	338.0	18.7	18.6	1.06
SHA	Ca. of ovary	4.2	0.6	3.7	3.10	14	13.00	780.0	55.8	53.0	1.05
FLE	Hodgkin's disease	4.8	0.3	6.1	5.8	23	27.80	1668.0	72.6	60.0	1.21
GRU	Ca. of testicle	5.8	0.55	4.5	4.0	14	23.20	1392.0	99.3	72.0	1.38
STU	Osteogenic sarcoma	4.8	5.0	6.9	1.9	6	9.12	547.8	91.2	60.0	1.52
ARO	Ca. of ovary	5.9	0.7	5.2	4.5	22	26.80	1590.0	72.3	44.0	1.64
MLR-4	Wilms's tumor	1.1	0.3	2.45	2.15	4	2.36	142.0	35.5	13.6	2.6
GUA	Ewing's sarcoma	1.4	0.9	5.4	4.5	7	6.30	378.0	54.0	16.8	3.21
MER-1	Wilms's tumor	1.1	0.1	3.0	2.9	4	3.19	192.0	48.0	14.3	3.35
CLA	Ca. of colon	3.6	2.25	5.7	3.5	3	12.5	750.0	250.0	45.0	5.54
				Patients with Granulocytic Leukemia							
MLS	AGL	4.2	2.3	19.0	16.7	11	69.5	4170	380.0	52.0	8.03
BUR-2	CGL-A	4.8	1.5	10.0	8.5	9	40.8	2448	272.0	60.0	4.53
BUR-1	CGL-A	5.2	5.5	25.0	19.5	15	101.4	6084	405.0	65.0	6.24
LUS	CGL	6.0	3.1	10.2	7.1	35	42.5	2550	72.9	75.0	0.97
FAL-1	CGL-A	3.2	8.0	27.0	19.0	13	60.8	3640	281.0	40.0	7.02
FAL-2	CGL-A	3.2	4.0	27.5	23.5	20	75.0	4500	225.0	40.0	5.64
LEE	CGL	4.0	3.3	68.6	65.3	56	262.0	524*	9.3	50.0	0.19
ROB	CGL-A	4.6	0.6	1.2	0.6	7	5.5	331	47.4	57.5	0.83
GOL	CGL	6.7	82.0	134.0	52.0	35	348.0	696*	19.9	83.8	0.24
LUT	AGL	3.8	4.2	24.8	20.6	9	79.0	4750	527.0	47.5	11.10

* Extracirculatory reservoir estimated as equal to the number of circulating blood cells

In 7 patients without hematologic abnormality, the intravascular residence times of the PMN, as determined by the method of hematodepression with CB-2348, were found to be 8.6 ± 1.7 and 13.6 ± 2.0 days.

In 19 studies on 10 patients with granulocytic leukemia, the IRT of the PMNs differed with the state of the leukemic process. In 5 patients with chronic granulocytic leukemia, an IRT_2 of 8.4 ± 1.6 days and an IRT_4 of 23.7 ± 3.7 days were found. In 3 patients with chronic granulocytic leukemia converting to an acute phase, an IRT_1 of 4.9 ± 0.4 days, IRT_2 of 8.9 ± 2.0 days, IRT_3 of 15.2 ± 1.6 days, and an IRT_4 of 18.6 ± 0.9 days were found. In primary acute granulocytic leukemia in 2 patients, PMN populations with an IRT_1 of 3.7 ± 1.06 days, an IRT_2 of 7.8 ± 0.50 days, and an IRT_3 of 13.6 days were found.

The minimal rate of production of PMNs in the patients without hematological abnormality, as measured from the intravascular residence time of the PMN, was 1.08 to 12.20×10^9/kg. body weight/day.

The rates of PMN production, as computed from the intravascular residence time in 10 patients with granulocytic leukemia on 19 studies, ranged from 0.17 to 10.48×10^9 PMNs/kg. body weight/day.

Approximate rates of PMN production, as determined by leukopheresis in 47 studies on 23 patients without hematological abnormality, were found to be 2.07 to 14.90×10^9/kg. body weight/day.

The rates of production of PMNs, as measured from the repopulation of the leukocyte reservoirs and peripheral blood following severe hematodepression, ranged from 0.86 to 5.54×10^9/kg. body weight/day.

By employing the technique of repopulation, the rates of PMN production in 10 studies on 8 patients with granulocytic leukemia were found to range from 0.19 to 11.1×10^9 PMNs/kg. body weight/day.

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this conversion, but effective leukopoiesis, resulting in PMNs, may actually be decreased

In any study of life span, it is essential to specify the cell type and period of maturation of the cell type being measured. This is of particular significance in the leukemic state.

The appearance of a short-lived PMN population in the more fulminant leukemias with a maintenance of the leukocyte concentration indicates that immature granulocytes will become evident. Whether the initiating pathogenesis of the leukemic state is the short-lived granulocyte evoking a compensatory leukopoiesis, or vice versa, has not been determined. It may explain, however, those situations in which there are high rates of granulocyte production with leukopenia In these aleukemic or subleukemic states, it is postulated that the rate of production of granulocytes is unable to compensate for the rapid rate of granulocyte destruction On the other hand, the prolonged life span of the PMN in chronic granulocytic leukemia compatible with a low rate of PMN production is suggestive of a compensatory mechanism

These data may explain why metabolic competitors, that is, antifolic acid and antiadenine compounds, have their greatest effect in the acute granulocytic leukemias in which the production rate and nucleoprotein synthesis are increased Usually, these same drugs are ineffective in the chronic granulocytic leukemias, where the rate of production and nucleoprotein synthesis may be normal or decreased However, there are occasional patients with acute granulocytic leukemia who fail to respond to these metabolic antagonists, just as there are patients with chronic granulocytic leukemia who, on occasion, derive dramatic benefit from these compounds Occasionally, the use of destructive agents such as X-ray therapy, HN2, TEM, and TEPA, may be harmful to patients with acute granulocytic leukemia in doses that further decrease the rate of leukocyte production, thus interfering with effective leukopoiesis which is already overtaxed

[illegible]

and initial granulocyte concentrations exceeding 62,000/cu. mm give ratios of 0 5 1 to 2·1 for the leukocyte reservoir to the number of circulating PMNs An average ratio of 1 1 was taken for those studies in which the initial granulocyte concentrations exceeded 62,000/cu mm Readjustment to more exact values may be made once the size of the granulocyte reservoir can be confirmed in a larger series of studies

Summary

Data on the rates of destruction and production of the postmitotic granulocyte, the PMN, were determined in 23 patients without primary hematological abnormality and in 10 patients with granulocytic leukemia

CROSS-CIRCULATION EXPERIMENTS IN ELUCIDATING THE VIABILITY AND DISTRIBUTION OF LEUKOCYTES*

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The dynamics of leukocyte production in man and experimental animals are poorly understood These studies were designed to make certain observations on the viability, distribution, and fate of transfused leukocytes by means of cross circulation in experimental animals Osgood[1] and others[2-5] have proposed the hypothesis that leukocytes in circulation represent but a fraction of the total leukocytes in the body The actual location of the noncirculating leukocytes remains a matter of speculation, but several studies[6-8] suggest that, under certain conditions, leukocytes may accumulate in large numbers along vascular walls. Craddock[2] has suggested that the bone marrow itself may contain a large portion of the available leukocyte "pool" The presence of vast reserves of mature lymphocytes in lymphatic tissue and

[illegible] f extensive noncircu-

[illegible] it is probable that

[illegible] cytes in circulation

One might predict that the introduction of a small number of viable leukocytes into the circulation of a severely leukopenic animal would result in rapid relocation of these cells into the depleted pools and a consequent rapid disappearance from circulation (FIGURE 1) A rapid rate of disappearance from circulation of transfused leukocytes, when viable leukocytes have been trans-

[illegible]

the peripheral blood leukocyte count (FIGURE 1) A schematic representation of the predicted relative disappearance rates of viable leukocytes trans-

[illegible]

of leukocytes and may result in leukocyte damage Peritoneal exudate leukocytes are abnormal in many respects, and the cells may receive additional injury during *in vitro* manipulation Human transfusions and cross-transfusion studies are subject to criticisms as to leukocyte damage, too few leukocytes, and immunological incompatibility In almost every instance cited, leukocyte circulation time has been very short and elevated leukocyte counts have not been sustained

In order to circumvent many of these criticisms, a method of temporary cross circulation in rats was employed This technique offers certain advan-

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include the necessity for anesthesia and the fact that primarily a lymphocyte population was being studied

The cross-circulation apparatus of Brodish and Long has been described in detail.[15] Briefly, the technique consists of placing polyethylene cannulae in a femoral artery and vein of the animals to be studied. Arterial blood flows freely from one animal through plastic tubes into the top of a small plastic cup. A measured volume (1 to 2 ml) is allowed to flow into the cup and, by

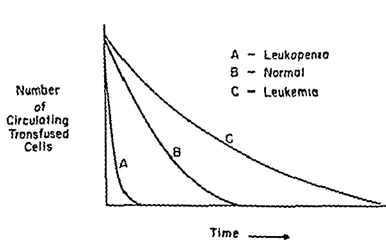
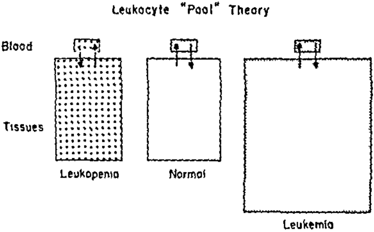


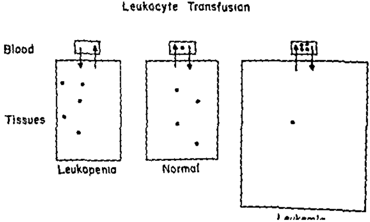
FIGURE 2. This schematic diagram indicates the relative disappearance rates from circulation of leukocytes transfused into recipients with varying degrees of leukocyte saturation

means of air pressure, is expressed from the bottom of the cup through polyethylene tubing into the vein of the opposite animal. Simultaneously, blood flows from the opposite animal in like manner. All experiments were performed with the animals anesthetized with Nembutal, 12 mg intraperitoneally and supplemented as necessary. To prevent clotting, both animals received 2 mg of heparin into the venous catheter at the beginning of cross circulation. Sprague-Dawley strain male rats weighing from 200 to 400 gm, obtained from a local breeder, were used in all experiments.

Radioiron mixing curves reported by Brodish and Long[15] showed that a complete mixing of the two circulations in rats of this size occurs after 25 to 30 ml of blood has passed from each animal. *One complete circulatory mixing* (30 ml) required 15 to 20 min. In most experiments, from 60 to 150 ml (2 to 5 mixings) was used, although we have found it possible to continue the cross circulation for as long as 10 total mixings (300 ml). At suitable intervals during cross circulation, blood was obtained from the arterial cannulae for total and differential leukocyte counts. After the cross

tages over previous methods of study The male albino rats used demonstrated no erythrocyte group incompatibility, which probably lessened the possibility of leukocyte immunological incompatibility. The blood was exposed briefly only to nonwetting surfaces, and the rate of leukocyte transfer





all were negative: (1) splenectomy of the irradiated recipient, (2) cortisone

sistent exponential disappearance of leukocytes in the recipient animals represents a redistribution or partition of the transfused leukocytes into the

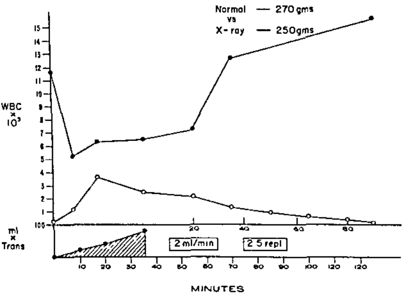


FIGURE 3 Leukocyte changes during and after cross circulation between a normal donor and an irradiated recipient rat The period of circulatory mixing is indicated by the diagonally striped area The leukocyte counts of the normal donor are designated by solid dots and those of the irradiated recipient by open circles

depleted noncirculating leukocyte reservoir In no instance were the curves linear, as one might expect if they represented leukocyte life span and death by senescence

Leukocyte counts in the donor rats after cross circulation was discontinued tended to remain at low normal levels during the period of observation, although some animals showed a gradual rise in count This response suggested either depletion of the readily available reserve or, possibly, only lack of specific stimulus to restore the circulating white cell volume to normal It should also be borne in mind that these animals, under prolonged anesthesia and cross circulation with a severely irradiated animal, may have been incapable of a truly physiological response However, Craddock *et al*,[18] using a direct method of leukocyte removal in dogs, noted a similar leukocyte depression and slow recovery.

circulation was discontinued, the arterial cannulae were retained in the artery for blood sampling.

In order to support the contention that leukocyte damage did not develop during cross circulation, the blood of 5 pairs of normal rats was exchanged for a total of 2 to 5 circulatory mixings. Total leukocyte counts, taken at approximately 10-min. intervals during the cross-circulation procedure and for a period of 90 min. postcirculation, showed no significant change from the precross-circulation levels. If mechanical disruption of leukocytes had taken place during cross circulation, leukopenia should have developed in these animals. In the course of these studies, it was noted that the blood from the tails of these rats was found to give a markedly variable leukocyte count and was not suitable for these studies. In normal rats the tail counts were found to be from 1.5 to 3 times higher than the simultaneous arterial samples. On the other hand, arterial and venous counts remained equal.

A second group of observations was made during and following cross-circulation between normal and leukopenic rats. In most instances, leukopenia was induced by X irradiation. A dosage of 800 r (200 kv., 15 mAmp., Thoreus filter, 50 cm. distance) was found regularly to produce arterial leukocyte counts of less than 400/cu. mm., 72 hours after irradiation, when all

count of the donor animals fell. These data indicated that the leukocytes transfused into the leukopenic animals were redistributed, sequestered, or

leukocytes mobilized appeared to be at least equivalent to the observed

circulating sites, a test of leukocyte function as an index of viability was employed. The test system was that of evaluating the efficiency of the transfused leukocytes in controlling an induced experimental bacteremia The actual experimental method has been described in some detail previously,[17] briefly, it consists of the injection of 200,000,000 viable *Escherichia coli* organisms through a polyethylene catheter placed in a femoral vein of the animal Blood samples taken from the femoral artery were obtained at inter-

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Six groups of animals were studied group 1, normal rats, group 2, lethally irradiated rats at 72 hours postirradiation, group 3, irradiated rats following cross circulation with normal rats, group 4, normal rats that had served as cross-circulation partners for irradiated rats of group 3, group 5, zymosan-treated rats, and group 6, irradiated rats subjected to replacement transfusion with normal rat blood from which the leukocytes had been removed partially by mechanical means Leukocyte counts of the pools of reconstituted bloods varied from 500 to 1700 cells/cu mm

The results of this study are indicated in FIGURE 5 As has been demonstrated previously[17] and as indicated in this figure, rats subjected to severe radiation are unable to remove injected *E coli* from their blood as efficiently as normal rats This defect in bacterial removal was improved significantly after cross circulation of irradiated rats with normal rats The beneficial results of the cross circulation indicated that the improvement resulted from a transfer of some substance in the normal blood to the irradiated rat Injections of zymosan in normal rats in an attempt to deplete the bactericidal

teremia, but the most probable explanation for this seemed to be incomplete leukocyte removal from the normal blood More probably, the transfused leukocytes were the source of the improvement noted after cross circulation, and the leukopenia of the irradiated rats was responsible for the high level of bacteremia in these animals This would indicate that the transfused leukocytes were not destroyed immediately and were capable of phagocytizing the *E coli* in circulation at a time when most of the leukocytes had disappeared from active circulation[18] Therefore, the rapid disappearance of

has been particularly evident in certain of the *in vivo* capillary microscopy studies of Vejlens[4]

Evidence for viability of the leukocytes transfused into the irradiated recipient gave additional support to the hypothesis that the blood survival time of transfused leukocytes might be increased if the pool of tissue leuko-

... consistent with the hypothesis outlined in ...

ments, the actual sites of redistribution are not known, and there is no

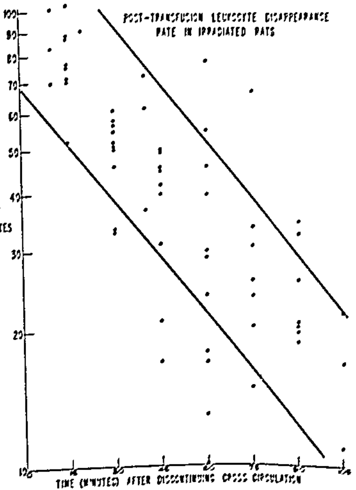


FIGURE 4. Fall of arterial blood leukocyte counts of irradiated recipient rats after cessation of cross circulation

direct evidence for persistence of leukocyte viability. The failure of leukopenia to develop in cross-transfused normal animals might suggest that these cells are not destroyed rapidly and remain viable in the contralateral recipients.

In order to test the hypothesis that the leukocytes transfused into the leukopenic recipient animals were not destroyed but remained viable in non-

were used for the sole purpose of providing a large donor leukocyte popula-

subjected to the cross-circulation procedure three days *postirradiation* In order to obtain adult animals with leukemia, suckling rats were inoculated

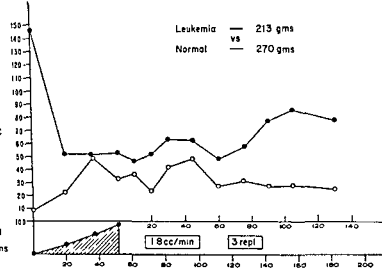


FIGURE 6 Leukocyte changes during and after cross circulation between a leukemic donor and a normal recipient rat The period of circulatory mixing is indicated by the diagonally striped area The leukocyte counts of the leukemic donor are designated by solid dots and those of the normal recipient rat by open circles

intraperitoneally with a splenic cell suspension from a leukemic donor animal Most of the animals developed leukemia when they weighed 100 to 150 gm Since they were too small for cross-circulation studies, the leukemia was

leukemic and normal animals, and a total of 7 cross circulations was performed between leukemic and X-ray leukopenic rats

When irradiated leukopenic rats were cross-circulated with leukemic animals, the arterial blood leukocyte counts of the irradiated animals were similar to those obtained when normal rats served as the leukocyte donors The total leukocyte counts of the leukemic donors dropped rapidly, but equilibra-

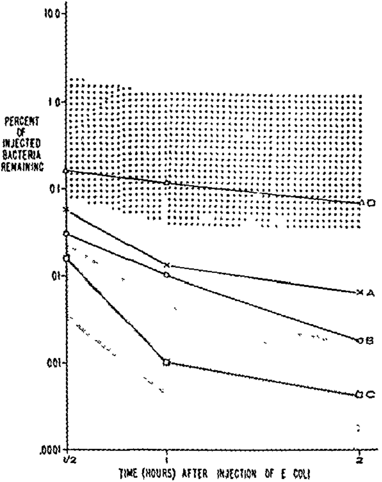


FIGURE 5 Mean percentage of injected bacteria remaining in the blood of rats is shown by a solid line for each of the following groups (*a*) cross circulated, irradiated rats, (*b*) normal donors following cross circulation with irradiated rats, (*c*) zymosan-treated normal rats, and (*d*) irradiated rats transfused with leukopenic blood The range of bacteremia levels for normal and irradiated rats that have not been cross circulated is represented by the heavily and lightly stippled areas, respectively

cytes was normally saturated and acted to impede circulatory loss In order to test this hypothesis, attempts were made, first, to saturate leukopenic animals with large volumes of leukocytes in order to retard the rate of peripheral leukocyte removal, and, second, to elevate greatly through cross circulation with leukemic rats the peripheral leukocyte counts of normal rats in order to demonstrate the ability in normally saturated reserves to effect more prolonged circulation of the transfused cells In these studies leukemic rats

The irradiated rats received 800 r total body air dose of X ray, and each was subjected to the cross-circulation procedure three days postirradiation In order to obtain adult animals with leukemia, suckling rats were inoculated

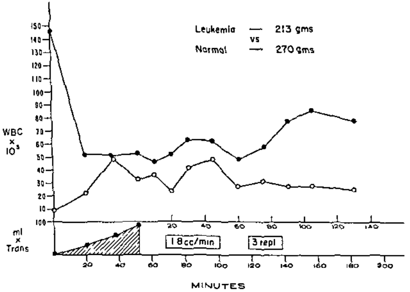
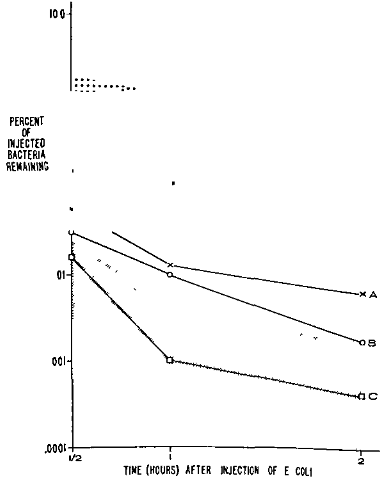


FIGURE 6 Leukocyte changes during and after cross circulation between a leukemic donor and a normal recipient rat The period of circulatory mixing is indicated by the diagonally striped area The leukocyte counts of the leukemic donor are designated by solid dots and those of the normal recipient rat by open circles

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increase occurred during the 90-min observation period after the procedure As compared to the normal donors, the leukocyte counts of the leukemic donor rats showed greater depression and less tendency to recover after cross circulation

The Shay chlorleukemia closely resembles human subacute or chronic granulocytic leukemia in its clinical and morphologic aspects It was of vital importance to note that the post cross-circulation survival rates of leukemic and normal leukocytes in X-ray leukopenic animals were identical This indicated that, under these experimental conditions, leukemic leukocytes of this type behaved and were handled in much the same way as were normal leukocytes Probably the fact that the transfused leukemic leukocytes disappeared from the circulation of normal rat partners at a much slower rate than from the circulation of irradiated rat partners is due to differences in size of the total body pool of leukocytes. The introduction of 5 to 10 times the normal number of leukocytes in the form of leukemic cells was insufficient to alter the equilibration rate between leukocytes in circulation and those in the depleted pools of the X-rayed animals On the other hand, the normally saturated leukocyte pools of the nonirradiated recipients allowed the entering leukocytes to remain in circulation for much longer periods of time The results of the cross-circulation studies are summarized in FIGURE 8 There was good evidence that the differences in the removal rates could not be explained on the basis of increased reticuloendothelial activity, local vascular factors, or an induced leukocytosis in the recipient animal

It is true that the normal rat contains a lower percentage of granulocytes in his peripheral blood than does either man or many other species However, the [illegible]

should apply to any granulocyte system, even though the number of cells involved may not be identical Again, these observations appear to conform with the general principles outlined in the pool hypothesis

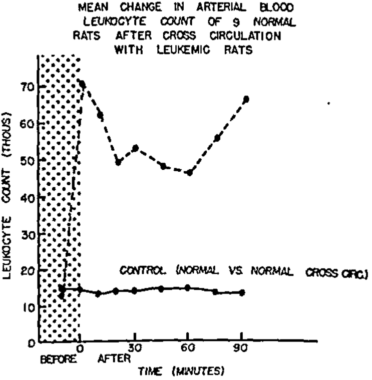
In order to shed some light on the problem of the location of the transfused leukocytes in noncirculating sites, more prolonged observations on the cross-transfused recipient animals were made Because of the small total number of leukocytes transfused, it seemed improbable that microscopic examination of tissue sections would permit the identification and localization of the transfused cells The unique circulation of the rat suggested that the capillaries and venules of the tail might be a site of intravascular leukocyte aggregation Therefore, certain studies of the normal rat tail were undertaken and the tail blood leukocytes were compared with the arterial blood leukocyte counts during and after various cross-circulation [illegible]

mal rats, simultaneous artery

tail blood leukocyte counts [illegible]

arterial counts Serial leukocyte counts were obtained from 5 successive drops of blood expressed from the tails of normal rats In all instances there was a precipitous fall in white count, mean values in 4 experiments decreasing

tion of leukocytes did not occur with continued cross circulation After the procedure was terminated, the irradiated rat count fell rapidly in an exponential fashion The rate of decrease of these leukemic cells in the irradiated rat appeared almost identical with the disappearance rate of transfused normal leukocytes *When normal rats were cross-circulated with* leukemic animals, the arterial blood leukocyte counts of the normal recipients



...creased greatly Immediately after cross circulation, the leukocyte

...

the normal animals. Following the ...

of the leukemic donor animals during cross circulation, only a moderate

that the white blood cells transfused by this method are not immediately destroyed within the recipient Again, these data are consistent with the pool hypothesis of leukocyte distribution, and they suggest that small venules, arterioles, and capillaries may be important sites in which noncirculating leukocytes aggregate out of active circulation

Summary

By means of cross-circulation experiments between normal, leukemic, and X-ray leukopenic rats, data are presented to support the contention that a large pool of noncirculating leukocytes exists. Indirect evidence is presented [illegible]

the body may be important reservoirs for noncirculating leukocytes

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approximately half the count of the initial droplet This information suggested that the tail vessels of the normal rat might act in retaining leukocytes in a fashion similar to that observed in certain capillaries of man.[8, 20] Following many cross-circulation experiments, the arterial and venous cannulae were removed, the vessels ligated, and the skin incisions closed. Total and

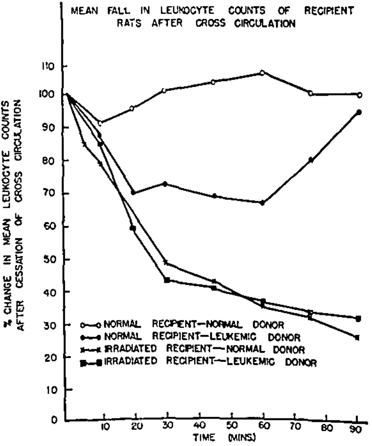


FIGURE 8 Mean percentage change in leukocyte levels of various recipient rats during 90 min observation after cross circulation

differential leukocyte counts were obtained daily thereafter for 3 to 6 days During the 3 days of observation it was evident that the tail counts of the irradiated rats after cross circulation with leukemic rats were markedly elevated as compared to those of the irradiated, untreated rats. In a study

REGULATION OF THE LEUKOCYTE LEVEL*

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Investigation of the life span and the elimination of leukocytes have received increasing attention since phases of impaired leukocyte elimination in leukemic patients were described. (Bierman *et al.*, 1949, 1951, 1952, 1955, Lanman *et al.*, 1950) Doan (1954) discussed the possibility that in some chronic leukemias the large number of mature and "overmature" leukocytes with increased life spans indicates a defect in elimination mechanisms. Osgood (1957) formulated a theory implicating factors that shorten leukocyte life span in leukemogenesis. A shortened life span would reduce the proportion of older, mature leukocytes and reduce the availability of hypothetical growth regulators or inhibitors originating from these cells, thus allowing unrestrained growth of the corresponding stem cells.

Little is known about the physiological regulation of the elimination of leukocytes. Until recently it was assumed that whatever regulation exists pertains to the production of leukocytes and their release into the circulation. We shall not attempt to survey the literature on the regulation of leukocyte levels in the peripheral blood, many reviews are available on this topic (Garrey *et al.*, 1935, Sturgis *et al.*, 1943, Fichtelius, 1953, Tullis, 1953, Ambrus, 1955, Bierman *et al.*, 1955, Yoffey, 1956, Lissac, 1957, and Ambrus and Ambrus, 1959). Instead, we shall summarize the studies of our own group on the regulation of leukocyte removal (Ambrus *et al.*, 1953, 1953*a*, 1953*b*, 1954, 1954*a*, 1954*b*, 1955, 1955*a*, 1956, 1956*a*, 1956*b*, 1957, and 1959) and present a preliminary report of unpublished work.

Methods and Materials

In vivo catheterization studies. In order to study leukocyte-removing functions, cardiac catheters were inserted into blood vessels leading into and out of the various organs of experimental animals. The catheters were prepared, and cardiac and pulmonary catheterization were performed under fluoroscopic guidance as described previously (Ambrus *et al.*, 1954*b*). Blood vessels of abdominal organs were catheterized after laparotomy by inserting a polyethylene catheter through a small side branch and ligating it into the branch, thus the flow in the main vessel was not impaired. Femoral arteries and veins were also catheterized through side branches. Hepatic veins were catheterized by passing cardiac catheters into the inferior vena cava and either directing their tips into hepatic veins or sampling caval blood at the level of the hepatic veins. In some experiments the vena cava was ligated.

Leukocytosis-promoting agents were used to investigate whether leuko-

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Comparing results obtained by different types of labeling may aid in evaluating sources of errors in the procedures Many of the errors of long-term cell survival studies (such as, reincorporation of the label) were avoided by restricting our study to short-term experiments.

Isolated organ studies. Heart-lung preparations of dogs were made as described previously (Ambrus *et al*, 1954*b*). Perfused lung preparations of mice were made in open chest animals under artificial respiration given

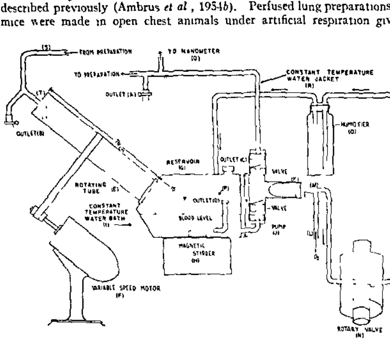


FIGURE 4 Diagram of mechanical heart-lung apparatus

through a tracheal cannula with the aid of a respiratory pump A polyethylene catheter was introduced through the jugular vein into the right ventricle The venae cavae were ligated A second polyethylene catheter was introduced into the left ventricle through the aorta and the aorta was ligated Oxygenated blood was perfused through the preparation without recirculation Leukocyte counts were compared before and after a single

cytes Liberation of serotonin and other toxic factors from the latter constricts hepatic venous sphincters and produces the phenomenon of "blue, hard liver" (Andrews and Maegraith, 1951) We found that oxygenation by "bubble," "foam," "spray," or "drop" oxygenators caused clumping of leukocytes and thrombocytes. This happened when blood was exposed to

sojourn of leukocytes in the circulation was less than 3 hours. This agrees with the estimates of many, but not all, investigators as to the length of the intravascular phase of the life span of leukocytes. FIGURE 3 shows the disappearance of labeled leukocytes in a dog following intravenous injection of 7 mg/kg of quinacrine. Following injection, most of the leukocytes of the animal became labeled, within 1 hour the majority of labeled cells disappeared and, after 2 to 3 hours, practically no fluorescent cells remained in the circulation, although an occasional labeled cell was discovered as late as

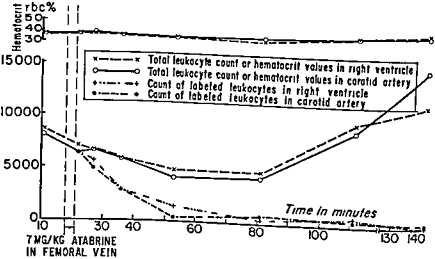


FIGURE 3. Dog cardiac catheterization. Disappearance of labeled leukocytes from the circulation of a dog following intravenous injection of 7 mg/kg of quinacrine (Atabrine).

48 hours after injection. Quinacrine-labeled leukocytes retained their fluorescence under ultraviolet light following centrifugation and washing with physiological saline.

The following tests were applied to determine whether the labeling procedures used might have affected the viability of the leukocytes: (1) Schrek eosin resistance test (Schrek, 1936, 1943), (2) ameboid motility on the warm stage microscope, (3) phagocytosis and opsonization (Boerner and Mudd, ... ctric ... (4) ... changes occurred in the above tests following P... or quinacrine labeling other than a decreased oxygen consumption after quinacrine. It has been shown that quinacrine inhibits cytochrome reductase, cytochrome oxidase, and glucose-6-phosphate dehydrogenase of mammalian tissues (Haas, 1944, Wright and Sabine, 1944). All labeling procedures ... e some effect on biological systems. ... consumption of leukocytes without ... phagocytosis, P... labeling introduces ionizing radiation into the cell

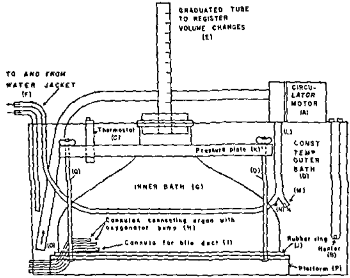


FIGURE 6 Diagram of chamber for isolated organs

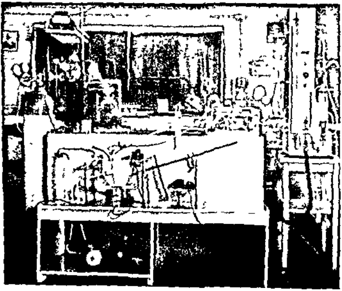


FIGURE 7 Mechanical heart-lung apparatus for the perfusion of isolated organs

oxygen in a discontinuous phase. On the other hand, oxygenation of a thin layer of blood in a continuous phase on a nonwettable surface caused no such change FIGURE 4 is a diagram of the apparatus used. The entire machine, except the rubber finger stall in the pump, was constructed from polyethylene Blood from the reservoir was pumped by a Dale-Schuster pump into the preparation A rotary valve directed oxygen from a cylinder alternately to the finger stall of the pump and to the humidifier From the latter, oxygen entered the reservoir and ascended through a rotating polyethylene tube to the outside Venous blood returning from the preparation flowed in a thin film on the inner walls of the rotating tube surrounding the oxygen that

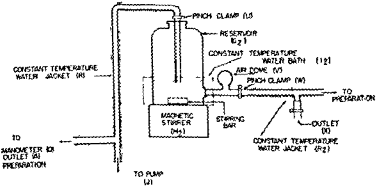


FIGURE 5 Diagram of apparatus for double perfusion of isolated livers.

ascended in the core The reservoir and all tubing were jacketed and perfused from a constant-temperature water bath The rotating tube was warmed by an infrared lamp Blood in the reservoir was kept in gentle motion by a magnetic stirrer In a later modification of the apparatus a rotor-type pump* was substituted for the Dale-Schuster pump, eliminating the rubber finger stall and allowing circulation through all plastic tubing.

Isolated livers were perfused through either the portal vein or the hepatic artery, or both For double perfusion, the apparatus shown in FIGURE 5 was used Arterial blood from the pump entered a T-tube, one branch of which led into the hepatic artery, the other into a reservoir from which blood entered the portal vein by gravity flow Thus the hepatic artery was supplied by pulsating flow at arterial pressures and the portal vein by continuous flow. The reservoir was placed in a constant-temperature bath and its contents were agitated gently by a magnetic stirrer Pressures and flow rates were maintained within physiological range

Perfused organs were kept in a double bath, as shown in FIGURE 6 The inner bath was made from the top of a 5-gal Pyrex bottle sealed with a pressure plate and screwed to a rubber latex ring resting on a Plexiglas platform This inner bath was submerged in a constant-temperature outer bath. Cannulae connected the blood vessels of the organ preparation to the heart-lung

* American Instrument Co , Rochester, N Y.

trates a Thiry-Vella loop segments of the small intestine are freed from the continuity of the intestinal tract and both ends brought out through the abdominal wall with their blood and nerve supply kept intact Animals were used for experiments ten days or more after surgery

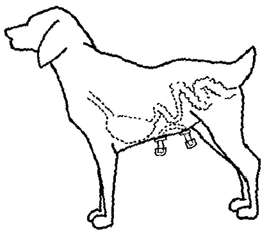


FIGURE 10 Schematic representation of Thiry Vella intestinal loop

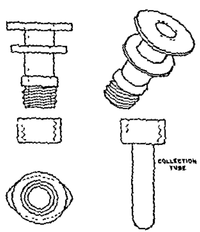


FIGURE 11 Construction of polyethylene cannulae and collection tubes for gastrointestinal fistulae

FIGURE 11 shows the construction of the polyethylene cannulae and collection tubes FIGURE 12 shows a cannula, collection tube, and cap The upper flange of the cannula was placed inside the fistula and the lower flange was tightened against the abdominal wall from the outside The cap that

machine. Other cannulae permitted the collection of bile flow from isolated livers (as a sign of functional activity of the organ) and the measurement of blood pressure in various blood vessels. A graduated tube extending from the top of the inner bath registered volume changes of the organ. The inner bath was filled with glycerol. FIGURE 7 shows the entire apparatus.

Blood samples were taken through side branches of the polyethylene tubing at points where blood entered and left the organ preparations Oxygen and

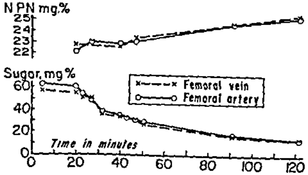


FIGURE 8 Isolated, perfused dog hind limb Blood sugar and NPN values in an isolated hind-limb preparation

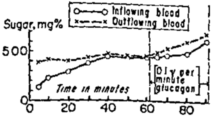


FIGURE 9 Dog liver perfused through portal vein Blood sugar values in an isolated liver preparation

carbon dioxide tensions of the blood were determined with the Van Slyke apparatus

liver preparation (FIGURE 9). All experiments were carried out within 2

or into both

cyte count and blood smear from the tail vein, and inspection of the abdominal organs after laparotomy under hexobarbital anesthesia If diagnosis was not confirmed histologically, the results were excluded from the series

Hematological techniques For the most part, routine hematological methods were used Blood was always handled in polyethylene containers or siliconeized glassware From blood vessels leading into and out of various organs, simultaneous blood samples were drawn into siliconeized syringes and discharged into silicone-coated tubes Differential counts were made from cover glass smears stained with Wright-Giemsa stain Centrifuged leukocyte concentrates from alveolar and gastrointestinal washing fluids were stained by the Papanicolaou method The following stain was developed for [illegible]

distilled water, solution 2 0 1 per cent eosin and 0 1 per cent Azo Phloxine in the same solvent as solution 1 Before use, solutions 1 and 2 were mixed in a 2 1 ratio The error of counting leukocytes has been calculated previously (Ambrus *et al*, 1954*b*) In these studies the 95 per cent confidence limits were between 11 and 16 per cent

Results

Removal of leukocytes by the pulmonary circulation Cardiac catheterization experiments were undertaken in anesthetized dogs by inserting catheters under fluoroscopic guidance into the pulmonary artery and vein or into the right chambers of the heart and into the carotid artery Preliminary experiments indicated no significant difference among leukocyte counts of the pulmonary vein, the left chambers of the heart, and the large arteries The prolonged presence of cardiac catheters in the heart or pulmonary vessels of anesthetized dogs caused fluctuations in the leukocyte count (usually leukopenia followed by leukocytosis). To avoid this error, all blood samples were obtained within 25 min after the initiation of anesthesia

FIGURE 13 shows results of a typical experiment Of sample pairs during a 20-min period, most counts were higher in the right ventricle than in the carotid artery, indicating leukocyte removal in the lungs The cells affected were chiefly polymorphonuclear neutrophils TABLE 1 presents the differences in total leukocyte counts in 9 to 10 pairs of right auricle and carotid artery samples taken from each of 13 dogs A highly significant difference was evident Similarly, TABLE 2 presents the differences in differential counts in 5 to 10 pairs of samples taken from each of 6 dogs Analysis of covariance for the differences in total counts and neutrophil counts indicated a dependence of the differences in total counts on the differences in polymorphonuclear neutrophils

TABLE 3 shows a series of experiments where single pairs of simultaneous samples were taken, either by catheterization or by direct puncture of the vessel, from vessels leading into or out from the pulmonary circulation Data were analyzed by "sign" test and by analysis of variance Because the number of samples was relatively small, the differences were not significant

closed the cannula throughout the day was replaced with the collection tube during experiments Thiry-Vella loops were perfused with warm physiological salt solution during the experiment; spontaneous secretions were collected from esophageal and gastric fistulae

To determine whether labeled leukocytes removed by the lungs do migrate into alveoli, "alveolar washing" experiments were undertaken. Dogs were exsanguinated through the carotid artery, a hemostat was placed on the trachea below the larynx, and the respiratory system was removed The lungs were immersed for a few seconds in warm (37° C.) saline to remove blood from the pleural surfaces A polyethylene funnel was ligated into the

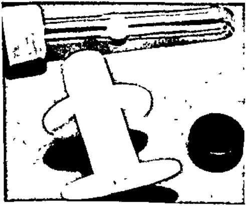


FIGURE 12 Polyethylene cannula, cap, and collection tube for gastrointestinal fistulae.

trachea and the lungs filled with warm saline, which was distributed throughout the lungs by gentle manual pressure applied alternately to various segments The entire system was inverted and the fluid drained into a siliconeized beaker This process was repeated three times. Further washings removed only very small numbers of residual leukocytes Volume, leukocyte counts, and erythrocyte counts of the washing fluid were determined. Samples with significant red cell counts were excluded from evaluation.

Animals and tumors Mongrel dogs of either sex weighing between 10 and 40 kg. were used Pentobarbital (Nembutal) anesthesia was given with an

The following [illegible] and C58 lymphatic leukemia [illegible]

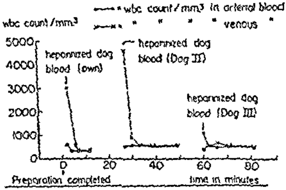


FIGURE 14 Regulation of circulating leukocyte level by the heart lung preparation of the dog

TABLE 1
MEAN DIFFERENCE IN TOTAL LEUKOCYTE COUNTS
Right Auricle Minus Carotid Artery

Dog No	No of Diff (Ni)	Diff *
1	10	+471
2	10	−460
3	10	+446
4	10	+1387
5	9	+1600
6	10	+1365
7	9	−217
8	10	+335
9	10	+538
10	10	+1390
11	9	+2467
12	10	+585
13	10	+730
Weighted mean diff N = 127		+807
Ninety five per cent spread		−2000 to +3600
Actual spread		−2300 to +4400
Ninety-five per cent confidence limits of mean diff		+334 to +1280
Significance of mean diff		p < 0.01

* Symbols + difference = right auricular count higher than count in carotid artery, − difference = right auricular count lower than count in carotid artery

ful blocking agent of these functions, was given to dogs 6 to 24 hours before establishing heart-lung preparations No impairment of leukocyte-removing ability occurred Menkin (1946, 1956) has reported that crude pyrexin and a leukopenic factor isolated from acid inflammatory exudates produced leukopenia by inducing accumulation of leukocytes in the alveolar walls of

in all series and with both types of analyses However, the differences became significant when all values of samples taken from locations leading into the lungs were considered together and compared with the similarly pooled values of samples obtained from locations leading out of the lungs.

Regulation of the leukocyte level in the heart-lung preparation. In intact animals where leukocyte populations are continuously renewed, it is difficult

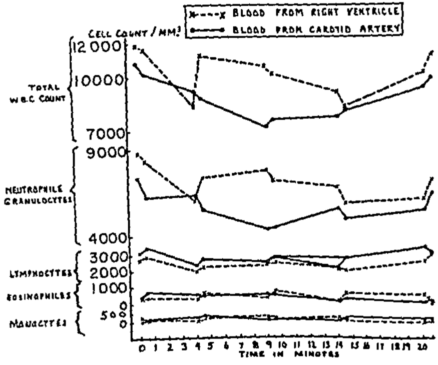


FIGURE 13 Dog cardiac catheterization Removal of leukocytes by the pulmonary circulation of the dog

to study the regulatory function of individual organs upon the leukocyte count In our isolated heart-lung preparations of dogs, rapid removal of neutrophil granulocytes occurred until a low level was reached. This level was maintained for the useful life of the preparation. When the blood was exchanged for a fresh sample, rapid removal of leukocytes again occurred until the same level was reached This level, constant for each individual preparation, varied between preparations from 500 to 1500 cells/cu. mm. Introduction of fresh blood into the preparation every 10 to 30 min for 3 hours did not exhaust the ability of the lungs to remove leukocytes A

the lungs, the sinusoids of the liver, and the splenic pulp From alkaline exudates a leukocytosis-promoting factor and leukopenin were isolated. Lyophilized powder of leukopenin, leukopenic factor, crude pyrexin, and leukocytosis-promoting factor were kindly given to us by V Menkin, Temple University, Philadelphia, Pa In doses of 20 mg, none of these substances altered the leukocyte-regulating activity of heart-lung preparations of dogs.

TABLE 3
AVERAGE LEUKOCYTE COUNTS IN VARIOUS BLOOD VESSELS OBTAINED BY SINGLE SIMULTANEOUS SAMPLING

Blood entering lungs V	Blood leaving lungs A	Significance of difference V A Analysis of variance	Significance of difference V-A Sign test
Vena cava superior 8135	Aorta 6727	$p < 0.01$	0, 6 N C
Right auricle 8953	Aorta 8114	N S	*1/12 $p < 0.01$
Right auricle 8032	Left auricle 7288	$p < 0.01$	*0 5 N C
Right ventricle 7002	Left ventricle 6018	N S	*0 5 N C.
Right auricle 13,065	Carotid artery 12,880	N S	†6/13 N S

Significance of the composite of all data marked with * 1/22 $p < 0.01$
Significance of the composite of all data marked with * and † 7/35 $p < 0.01$
N S = not significant, N C = not conclusive

Fate of leukocytes in the lungs Experiments were undertaken to investigate the fate of leukocytes removed from the circulation by the lungs Are these cells decomposed? Are they sequestered in pulmonary capillary beds? Can they return into the circulation? Do they migrate into the alveoli? What is their ultimate fate?

An experiment demonstrating the effect of epinephrine in releasing leukocytes from the pulmonary circulation is shown in FIGURE 1. FIGURE 15 shows an experiment where 500 ml of quinacrine-labeled blood was infused

culation occurred as indicated by rise of the total as well as of the labeled cell counts of the carotid artery samples This was followed by a secondary increase in the right ventricular counts The latter may be due to release of leukocytes from other organs as well as to the arrival of cells in the venous blood from the pulmonary circulation The number of labeled leukocytes

TABLE 2
MEAN DIFFERENCE IN WHITE BLOOD CELL COUNTS
Right Auricle Minus Carotid Artery

Dog no	No of differences	Type of count and difference				
		Total	P	L_y	F_0	M_0
1	10	+471	+51	+262	+65	+60
2	6	−300	−57	−58	−42	−45
3	5	+740	+250	+432	−400	+287
4	10	+1377	+1677	−378	+13	+1
5	5	+1640	+1052	+368	+195	−30
6	5	+594	+790	−254	+319	+20
Weighted mean diff		+769	+668	+29	+25	+45
Ninety-five per cent spread*		−1600 to +3200	−1800 to +3200	−870 to +930	−1000 to +1000	−300 to +400
Actual spread		−2100 to +3480	−2150 to +3480	−800 to +1200	−2000 to +1688	−284 to +439
Ninety-five per cent confidence* limits of mean diff		+50 to +1500	−100 to +1500	−320 to +380	−150 to +200	−70 to +160
Significance of mean diff		$p < 0.05$	$p < 0.10$	N S	N S	N S

* Rounded off values P = polymorphonuclear neutrophils, L_y = lymphocytes, E_0 = eosinophils, M_0 = monocytes

present in alveolar washings of untreated dogs, it must be assumed that either (1) labeled cells became concentrated in a small volume of alveolar fluid and lost some quinacrine to neighboring unlabeled cells, (2) small amounts of free quinacrine present in plasma were excreted selectively into the alveolar fluid and there reached sufficient concentrations to stain additional leukocytes, or (3) some labeled leukocytes disintegrated in the lungs and their label became incorporated into unstained cells

TABLE 4
TOTAL NUMBER OF CELLS IN LUNG WASHINGS, EXPRESSED IN MILLIONS

Control	Time after transfusion of 500 ml dog blood				Time between transfusion of 500 ml dog blood and injection of 20 μg /kg l-epinephrine Dog sacrificed 20 min later	
	5 to 10 min	11 to 30 min	2 to 3 hours	6 hours	2 to 3 hours	6 hours
420	1120	1406	1774	5809	1934	4810
513	1298	1480	2910	6480	2452	5164
864	1400	1506	3500	6650	3990	5510
1230	1472	1749	4320	7000	4020	7310
1311	1590	1814	5364	10749	4886	11390
1460	1600	1904	9617	12386	4951	20820
4200	8626	13240	12970	24300	7633	21427
9267	9620	21830	23960	45300	10794	26263
23560	11780	43727	24500	36830	25400	29312
26600	71490	51780	92775	97490	161300	72516
Mean 6942 5	10 999 6	14 043 6	18,169 0	23 319 4	22 756 8	20 452 6
Standard error of mean 3146	6659	6055	8667	8882 1	15 250 3	6339 8
Sign diff compared to the control group	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p < 0.01$		
Sign diff compared to the nontreated group sacrificed the same time after transfusion					$p > 0.05$	$p > 0.05$

In experiments where the lungs were removed 20 min after injection of 20 μg /kg l-epinephrine, the usual release of leukocytes from the pulmonary circulation occurred, however, no difference was found between the alveolar leukocyte population of these animals and those of animals sacrificed according to the same schedule, but not receiving epinephrine (TABLE 4). To compare the two independent pairs of means, two-sided "Student t" tests were utilized

int
aft

DNA-specific activity in the organs of recipient mice was expressed as labeled leukocytes per gram organ Of course, viable and disintegrating leukocytes could not be differentiated by this method A large number of leukocytes appeared to be taken up by the lungs, spleen, liver, and thymus. Three

became temporarily almost ... tion of the transf

In a series of ... quinacrine-labeled dog blood was infused intravenously into groups of 10 dogs. After various time periods the groups were sacrificed and the alveolar washing technique described above was applied. Results are summarized in TABLE 4. In control dogs, which did not receive transfusions, significant numbers of leukocytes could be washed out from the alveoli. These were chiefly histiocytes (or alveolar

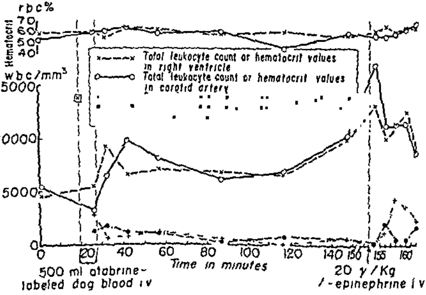


FIGURE 15. Dog cardiac catheterization. Reappearance of labeled leukocytes in the circulation following epinephrine injection.

macrophages), granulocytes, and lymphocytes. There was a remarkable paucity of columnar ciliated cells from the bronchial muco... found in human bronchial wash... larger cells ...

... level was not reached until 6 hours after infusion. A logarithmic transformation ... on the data and the ... tribution. The vari... and the pooled vari... differences. To compare treatments with control, the multiple comparison procedure of Dunnett (1955) was employed. One-sided comparisons were used.

Unfortunately, after transfusion with labeled cells almost all cells in the alveolar washings exhibited fluorescence. Since normal leukocytes were

after infusion, labeled leukocytes were identified in saliva collected through esophageal fistulae. Labeled cells in various stages of decomposition were found in fluid collected from gastric fistulae. Most of these cells appeared to be granulocytes. Because of the dependence on secretory rates and the

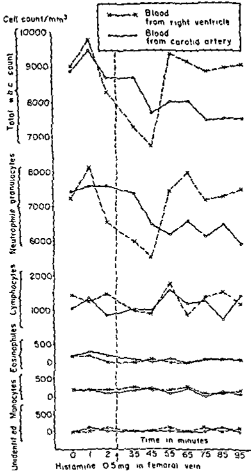


FIGURE 16. Dog cardiac catheterization. Effect of histamine on the removal of leukocytes in the pulmonary circulation.

difficulties in morphologic identification (particularly in the case of gastric secretions), these studies were essentially qualitative. However, some granulocytes apparently migrated from the alveoli up the tracheobronchial tree, and were swallowed and eliminated through the gastrointestinal tract.

hours after transfusion there was a decrease of activity in the lungs and spleen, and a notable increase in the thymus and bone marrow. In this respect there was no difference between normal and leukemic animals except that in controls, but not in leukemic animals, the liver appeared to have gained some leukocytes between 1 and 3 hours after infusion. The significance of this difference was questionable.

TABLE 5
NUMBER OF TRANSFUSED P^{32}-LABELED WBCs PER GRAM ORGAN IN RECIPIENT ANIMALS, CALCULATED ON THE BASIS OF DNA SPECIFIC ACTIVITY. EACH GROUP, 5 MICE
Donor Blood from Leukemic AK Mice

Organs	Recipients normal AK mice	
	1 hour after infusion	3 hours after infusion
Lung	246×10^6	148×10^6
Liver	130×10^6	457×10^6
Spleen	516×10^6	238×10^6
Thymus	173×10^6	356×10^6
Bone marrow	0	341×10^6
	Recipients leukemic AK mice	
	1 hour after infusion	3 hours after infusion
Lung	169×10^6	92×10^6
Liver	580×10^6	567×10^6
Spleen	367×10^6	98×10^6
Thymus	241×10^6	827×10^6
Bone marrow	0	356×10^6

These experiments seem to indicate that leukocytes are removed by the pulmonary circulation, where they probably marginate to the capillary walls. Some leukocytes eventually may re-enter the circulation (particularly under the effect of agents that produce hemodynamic changes in the pulmonary circulation), others migrate slowly through alveolar membranes into the [illegible]

[illegible] (thymus).

In order to f[illegible] removed by the lungs and migrated [illegible] esophageal or gastric fistulae were g[illegible] blood. Several hours

rine from the adrenals, and Staub (1946) demonstrated that epinephrine injection released histamine from the tissues The nervous and humoral factors regulating pressure-flow relationships in the pulmonary circulation, hormones (epinephrine, cortical steroids), and tissue hormones (histamine, serotonin) are probably involved in modifying the leukocyte level-regulating function of the lungs

Removal of leukemic leukocytes by the lungs of normal and leukemic animals No difference was found between the distribution of P^{32}-labeled leukemic

TABLE 7
CELL COUNTS/CU MM

Donor animals and leukocyte count of pool	Recipient animals and leukocyte count after passage through pulmonary circulation	
Leukemic AK	Normal AK	Leukemic AK
13,200	6500 4300	4700 3900
Leukemic C58	Normal C58	Leukemic C58
11,700	5500	6300
Leukemic F	Normal F	Leukemic F
39,000	9000	7600
Leukemic DBA/2	Normal DBA/2	Leukemic DBA/2
18,000	8500	6000

leukocytes in normal AKR mice and that in animals suffering from spon-
s of an experi-
was perfused
vith leukemia
C1498 Similarly, blood from C57/BL6 mice with leukemia C1498 was perfused through the lungs of normal DBA/2 mice and animals with leukemia P1534 Analysis of correlation coefficients indicated no difference between initial counts and filtration rates (findings similar to those in dog heart-lung preparations)
leukemic lungs i
experiments in v
taneous leukemia and from DBA/2 mice with methylcholanthrene-induced leukemia was perfused through the lungs of normal and leukemic recipients of the same strain There were no apparent differences between removal rates of normal and of leukemic lungs In contrast to the dog heart-lung

Effect of hormones on the regulation of leukocyte levels by the lungs. FIGURES 1 and 15 show release of leukocytes from the pulmonary circulation following epinephrine injection. Bierman *et al.* (1952, 1953*a*) demonstrated that histamine increased the pulmonary sequestration of leukocytes, and that this

TABLE 6
CELL COUNTS/CU MM

Blood from DBA/2 mice with P1534 leukemia	Transfused through lungs of normal C57/6 mice	Filtration (percentages)	Transfused through lungs of C57/6 mice with C1498 leukemia	Filtration (percentages)
22,400	8000	64	7650	67
32,000	11,000	66	14,200	56
12,000	5100	57	3000	75
14,000	6200	56	9360	33
11,000	4200	62	4500	59
13,500	3800	72	4000	70
10,500	2600	75	1900	82
Mean percentage		64.5		63.2
Significance of difference between filtration percentages				N.S.
Blood from C57/6 mice with C1498 leukemia	**Transfused through lungs of normal DBA/2 mice**	**Filtration (percentages)**	**Transfused through lungs of DBA/2 mice with P1534 leukemia**	**Filtration (percentages)**
42,000	11,000	74	9000	79
11,300	2900	74	3300	71
10,100	3350	67	2600	74
12,500	2600	79	4700	62
12,300	3150	74	2540	79
10,400	2200	79	5100	51
Mean percentage		74.5		69
Significance of difference between filtration percentages				N.S.

effect was less pronounced in leukemic patients (Bierman *et al.*, 1956). FIGURE 16 shows results of a similar experiment in a dog in which injection of histamine initially caused leukopenia; the leukocyte count of the carotid artery rose to a level temporarily above that of the right ventricle, followed by a period of increased pulmonary leukocyte removal.

Complex regulatory factors may be involved in this phenomenon. Dale (1906, 1920) showed that histamine injection caused pouring out of epineph-

rine from the adrenals, and Staub (1946) demonstrated that epinephrine injection released histamine from the tissues. The nervous and humoral factors regulating pressure-flow relationships in the pulmonary circulation, hormones (epinephrine, cortical steroids), and tissue hormones (histamine, serotonin) are probably involved in modifying the leukocyte level-regulating function of the lungs.

Removal of leukemic leukocytes by the lungs of normal and leukemic animals. No difference was found between the distribution of P^{32}-labeled leukemic

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13,200	4300	3900
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11,700	5500	6300
Leukemic F	Normal F	Leukemic F
39,000	9000	7600
Leukemic DBA/2	Normal DBA/2	Leukemic DBA/2
18,000	8500	6000

leukocytes in normal AKR mice and that in animals suffering from spontaneous lymphatic leukemia (TABLE 5). TABLE 6 shows results of an experiment in which blood from DBA/2 mice with leukemia P1534 was perfused through the lungs of normal C57/BL6 mice and animals with leukemia C1498. Similarly, blood from C57/BL6 mice with leukemia C1498 was perfused through the lungs of normal DBA/2 mice and animals with leukemia P1534. Analysis of correlation coefficients indicated no difference between initial counts and filtration rate

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leukemia was perfused through the lungs of normal and leukemic recipients of the same strain. There were no apparent differences between removal rates of normal and of leukemic lungs. In contrast to the dog heart-lung

preparations, large numbers of lymphocytes seemed to be sequestered by the perfused mouse lungs

Removal of leukocytes by the gastrointestinal tract FIGURE 17 shows results of an experiment in which 200 ml blood with quinacrine-labeled and P^{32}-labeled leukocytes was infused intravenously into a dog with a Thiry-Vella intestinal loop The loop was perfused with warm physiological salt solution

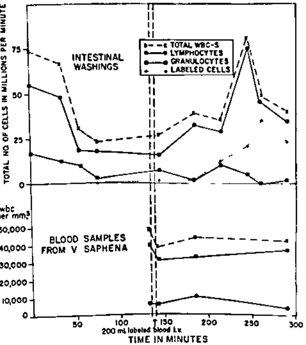


FIGURE 17 Elimination of leukocytes through Thiry-Vella intestinal loops following intravenous transfusion of labeled blood

washings were mononuclear, few granulocytes were seen Following blood transfusion, lymphocyte counts of the washings gradually increased and labeled cells appeared, reaching maximal levels about two hours after transfusion

In a similar experiment, 70 ml. labeled thoracic duct lymph was infused intravenously into a dog with a Thiry-Vella loop Results are shown in FIGURE 18 A rapid increase followed by a gradual decline in the labeled cells of the perfusate was noted

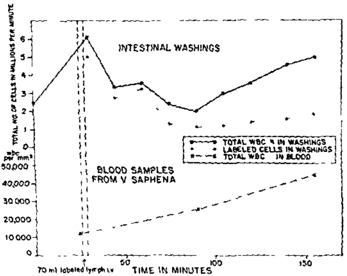


Figure 18 Elimination of leukocytes through Thiry-Vella intestinal loops following intravenous infusion of labeled lymph

Table 8
Number of Labeled Cells in Thousands ± Standard Deviation in Intestinal Loop Washings of AK Mice Obtained within 8 Hours After IV Infusion of 3.5 × 10⁸ Labeled Cells Each Group, 5 Mice

Donor	Recipient	
	Normal	Leukemic
Normal	620 ± 217	770 ± 423
Leukemic	470 ± 228	600 ± 256

Analysis of variance between the four groups $p > 0.05$.

lymphatic leukemia were used, no differences were seen in white cell elimination between these two groups of animals

Role of abdominal organs and peripheral capillary beds in the regulation of the leukocyte count Experiments were undertaken to study the possible role of organs other than the lungs and the gastrointestinal tract in the regulation

of leukocyte levels FIGURE 19 shows the results of such an experiment Quinacrine-labeled blood was infused into a recipient dog and the following blood vessels were catheterized subsequently splenic vein; portal vein, prior to the entrance of the gastrosplenic vein, vena cava inferior, below the renal vein and at the level of the hepatic veins, and carotid artery. Splenic contraction usually followed laparotomy in the dog and resulted in a pouring out

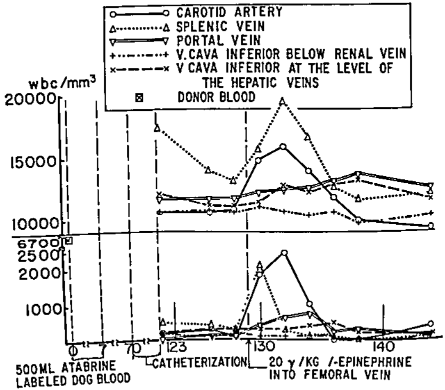


FIGURE 19 Dog *in vivo* catheterization Fate of leukocytes in the portal circulation and effect of epinephrine

of leukocytes into the splenic vein. This response gradually decreased and splenic vein counts approached or fell below splenic artery counts, indicating that under normal conditions the spleen probably removes leukocytes Portal vein counts usually were higher than arterial counts and vena cava inferior counts, either those below or at the level of the hepatic veins. The difference of the counts in the portal vein and the vena cava inferior at the level of the hepatic veins may be due to removal of the leukocytes by the liver and to dilution of portal blood with arterial and caval blood Within two hours after transfusion, most labeled cells disappeared from the circulation Intravenous injection of epinephrine caused immediate splenic contraction and a renewed increase in the total as well as the labeled leukocyte counts in the

splenic vein There was a rapid, pronounced increase in the leukocyte counts (labeled and unlabeled) in the carotid artery, indicating release from the pulmonary circulation No change was noticed in the vena cava inferior below the hepatic veins. Apparently peripheral capillary beds do not contribute significantly to the leukocytosis There was a gradual increase in the portal vein counts, probably indicating release from the splanchnic circulation In spite of the large increase in carotid artery and splenic vein counts and the moderate increase in portal vein counts (prior to entrance of gastrosplenic vein), there was only a slight increase in total counts and no increase in labeled counts in the vena cava inferior at the level of the hepatic veins This may indicate hepatic filtration of leukocytes Catheterization experiments were undertaken to investigate the fate of leukocytes in the hepatic and splenic circulation To study the role of the splenic circulation was

spleen sequesters all types of leukocytes, that many are decomposed, and that others are available to re-enter the circulation

Various types of experiments were designed to study hepatic leukocyte removal

(1) In the first series of experiments, catheters were inserted into the portal vein and the vena cava inferior at the level of the hepatic veins The hepatic artery and the vena cava inferior above the phrenicoabdominal vein were ligated A series of blood samples was taken within 20 min after ligation FIGURE 20 shows the results of such an experiment Portal vein

hepatic release of leukocytes occurred Data concerning normal leukocyte

garithmic

garithmic

Differ-

ences were significant at the 1 per cent level for all logarithmic cell counts except the "other and unidentified" group

(2) In a second series of experiments, blood samples were obtained from the hepatic artery, portal vein, and the vena cava inferior at the level of the hepatic veins The vena cava inferior was ligated above the phrenicoabdominal vein Preliminary experiments indicated that under these conditions the hepatic artery contributed about 10 per cent of the total hepatic flow The cell counts of the portal vein and hepatic artery were combined in a ratio of 9 1 and compared with the cell counts of the vena cava inferior at the level of the hepatic veins Results are shown in TABLE 10 The mean logarithmic difference for each cell type was tested for significance by means of Student t tests Significant differences were detected for the total cell, neutrophil, and lymphocyte counts

(3) In the third series of experiments, catheters were inserted into the

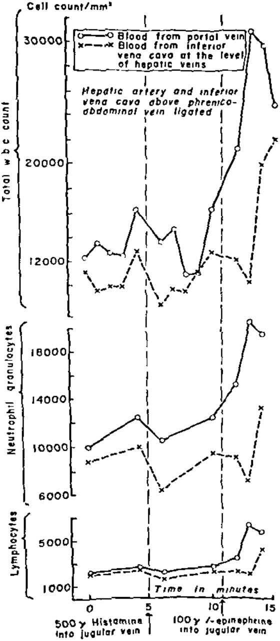


FIGURE 20 *In situ* dog liver catheterization Removal of leukocytes in the he[...] circulation

TABLE 9

REMOVAL OF LEUKOCYTES BY THE PORTAL CIRCULATION OF DOGS PORTAL VEIN AND VENA CAVA INFERIOR AT THE LEVEL OF HEPATIC VEINS CATHETERIZED, HEPATIC ARTERY AND VENA CAVA INFERIOR BELOW THE LEVEL OF HEPATIC VEINS LIGATED

Dog No	No of paired samples/dog	Total count			Neutrophil granulocytes		
		Mean diff $i - o$†	Mean log diff	SE of mean log diff	Mean diff $i - o$†	Mean log diff	SE of mean log diff
1	4	+1065	+0 0625	0 0356	+302	+0 0494	0 1892
2	14 (7)*	+5111	+0 2110	0 1011	+5049	+0 1834	0 1430
3	13	+7771	+0 1317	0 0536	+5285	+0 1403	0 1049
4	13	+11692	+0 2854	0 0536	+8889	+0 2936	0 1049
Means and standard error significance (V A)		+7474	+0 1961	0 0292 $p < 0 01$	+5968	+0 1925	0 0622 $p < 0 01$
		Lymphocytes			Eosinophils		
1	4	+675	+0 0955	0 0668	−70	−0 08833	0 9011
2	7	+1126	+0 1296	0 0505	+476	+1 5985	0 6812
3	13	+1360	+0 0671	0 0371	+947	+2 5994	0 4999
4	13	+1791	+0 2056	0 0371	+432	+0 2214	0 4999
Means and standard error significance (V A)		+1393	+0 1307	0 0219 $p < 0 01$	+26	+1 2839	0 2963 $p < 0 01$
		Monocytes			Other and unidentified cells		
1	4	+137	+0 2775	0 1254	+20	+0 1263	1 1230
2	7	+413	+0 1997	0 09482	+282	+0 3030	0 8489
3	13	+443	+0 2791	0 0696	+191	+0 1694	0 6772
4	13	+596	+0 4040	0 0696	+203	+1 5765	0 7487
Means and standard error significance		+460	+0 3078	0 04123 $p < 0 01$	+193	+0 6025	0 4034 $p > 0 05$

* In parentheses the number of samples is indicated in which differential counts were taken if this number is less than the number of samples in which total leukocyte counts were established

† Portal vein counts were designated inflowing cell counts (i) Counts from the inferior vena cava at the level of hepatic veins were designated outflowing cell counts (o)

hepatic artery and the portal vein, and the tip of a curved catheter was directed into a single hepatic vein Portal vein and hepatic artery counts were combined in a ratio of 9 1 and compared with the counts of the hepatic vein Results are summarized in TABLE 11 Analysis of data was undertaken as in the previous series Significant differences were detected for the

of all capillary beds or is restricted to specific organs, the femoral artery and vein were catheterized in four dogs Results are shown in TABLE 12, the analysis of data undertaken as before No significant difference was detected for any of the cell types

TABLE 12
LEUKOCYTE COUNTS IN THE FEMORAL ARTERY AND VEIN OF DOGS

Dog No	No of paired samples dog	Total count			Neutrophil granulocytes		
		Mean diff V-A	Mean log diff	S E of mean log diff	Mean diff V-A	Mean log diff	S E of mean log diff
1	7	+371	+0 02929	0 0258	+279	+0 04677	0 0480
2	2	+3145	+0 14283	0 0482	+130	+0 07890	0 0899
3	3	−2670	−0 12309	0 0394	−1850	−0 13110	0 0734
4	3	+3	+0 00011	0 0394	−60	−0 0051	0 0734
Means and standard error		+59	+0 008118	0 01744	−235	−0 00594	0 0330
significance (V-A)				p > 0 05			p > 0 05
		Lymphocytes			Eosinophils		
1	7	+114	+0 04224	0 0287	+144	+0 06570	0 6134
2	2	+445	+0 08200	0 0537	+270	+0 1110	1 1476
3	3	−300	−0 05325	0 0439	+3	+0 0116	0 9370
4	3	0 0	−0 00480	0 0439	+20	+0 1004	0 9370
Means and standard error		+53	+0 019023	0 0196	+108	+ 0678	0 4190
significance (V-A)				p > 0 05			p > 0 05
		Monocytes			Other and unidentified cells		
1	7	+10	−0 01932	0 3147	−1	−0 01384	0 4237
2	2	+240	+2 3349	0 5888	+55	+1 87018	0 7334
3	3	−90	−0 2239	0 4807	−23	−0 1119	0 5387
4	3	−17	−0 0698	0 4807	0	0	0
Means and standard error		+36	+0 024355	0 215	+8	0 22051	0 3051
significance				p > 0 05			p > 0 05

Leukocytes in the isolated hind-limb preparation Isolated hind limbs of dogs were perfused with the heart-lung machine described under *Methods.*

Epinephrine may liberate some preformed leukocytes from the bone marrow In addition, it is possible that in capillary beds there is a balance between constantly marginating and demarginating granulocytes, resulting in equal leukocyte counts in the inflowing and outflowing blood Hemodynamic changes temporarily may alter this balance as, for example, in the case of epinephrine injection when increased demargination is induced

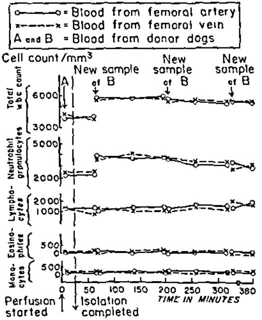


FIGURE 21 Dog hind limbs perfused through femoral artery Leukocyte counts in the isolated hind limb preparation

Regulation of the leukocyte count by the isolated liver Isolated liver preparations were maintained with the artificial heart-lung apparatus described above FIGURE 22 [illegible]

of the blood introduced into the preparation The leukocyte disappearance curves resemble those of the isolated heart-lung preparations (FIGURE 14) Levels attained in the liver preparations ranged from 500 to 1500 cells/cu mm, similar to those of the lung preparations However, there were several differences [illegible]
level usually [illegible]
30 to 45 min
phil granuloc[illegible]
cells also were filtered out

FIGURE 24 illustrates an experiment in which epinephrine and histamine were introduced into the preparation As in the *in situ* liver, these agents did not affect the leukocyte counts in the preparation.

Regulation of the leukocyte count by the isolated spleen It proved to be extremely difficult to work with isolated dog spleen preparations, since splenic

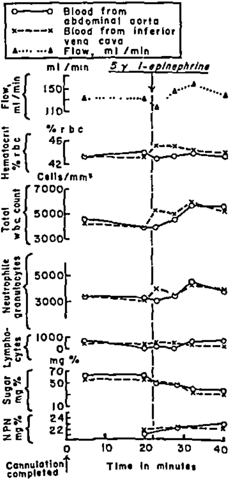


FIGURE 22 Isolated dog hind limbs perfused through abdominal aorta Effect of epinephrine on the leukocyte counts in the isolated hind-limb preparation

contraction due to the operative procedures interfered with the experiment. ... informative experiments could be performed FIGURE 25 shows

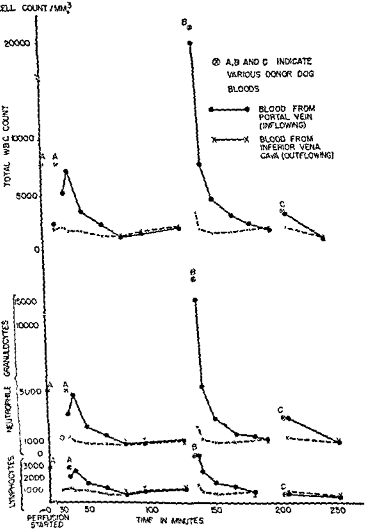


FIGURE 23 Isolated dog liver perfused through portal vein Regulation of leukocyte counts by the isolated liver

cytes, lymphocytes, eosinophils, and monocytes Large numbers of degenerating cells were released occasionally by the spleen in such experiments.

Discussion

It appears that leukocytes are removed from the circulation through the lungs and intestines, that they are removed and probably disintegrated in

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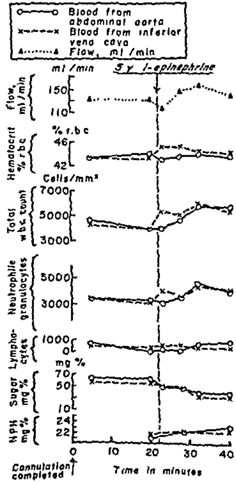


FIGURE 22 Isolated dog hind limbs perfused through abdominal aorta Effect of epinephrine on the leukocyte counts in the isolated hind-limb preparation

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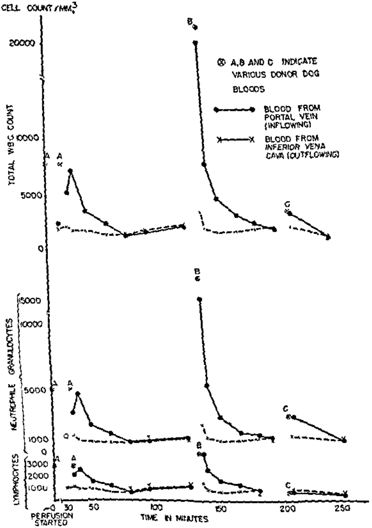


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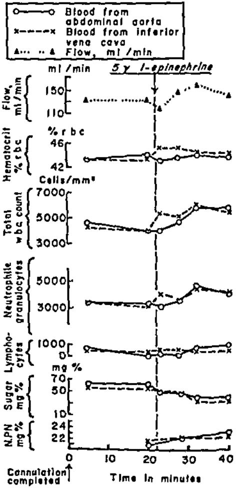


FIGURE 22 Isolated dog hind limbs perfused through abdominal aorta Effect epinephrine on the leukocyte counts in the isolated hind-limb preparation

contraction due to the operative procedures interfered with the experime Only a few informative experiments could be performed FIGURE 25 sho that after the spleen returned to a relaxed status it removed all types of wh cells, as did the liver FIGURE 26 illustrates results of an experiment in wh epinephrine and histamine were introduced into the preparation Bo agents caused splenic contraction and outpouring of neutrophil granu

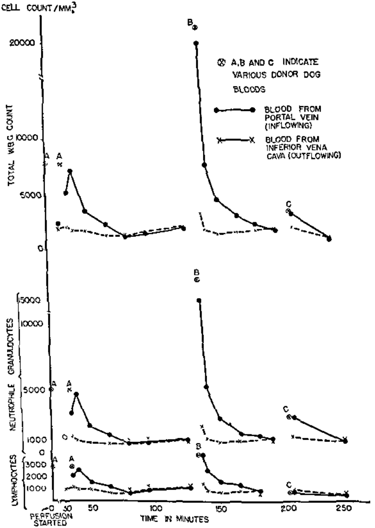


FIGURE 23 Isolated dog liver perfused through portal vein Regulation of leukocyte counts by the isolated liver

cytes, lymphocytes, eosinophils, and monocytes Large numbers of degenerating cells were released occasionally by the spleen in such experiments

Discussion

It appears that leukocytes are removed from the circulation through the lungs and intestines, that they are removed and probably disintegrated in

the liver and spleen, and that they are sequestered in lymphoid tissue and bone marrow with a destination not yet fully understood. In the isolated lung, liver, and spleen a definite regulatory function is evidenced. These

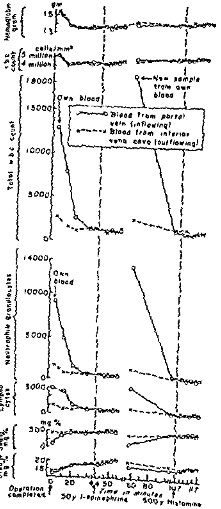


FIGURE 24 Isolated dog liver perfused through portal vein Effect of ep[illegible] histamine on the regulation of leukocyte count by the isola[illegible]

the white cell count begins to rise and eventually becomes higher than the starting value These authors interpreted their findings as follows, "The inability to reduce this 'floor' results from an increased rate of entry of leukocytes during this period (leukopheresis) The failure of the leukocyte

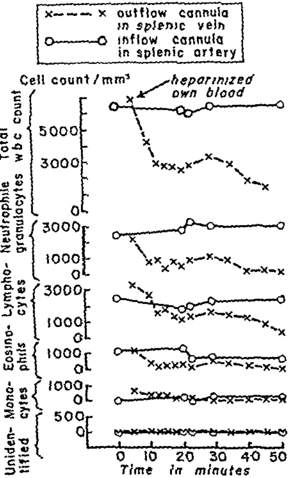


FIGURE 25 Removal of leukocytes by the isolated dog spleen

count to begin to rise immediately after cessation of the procedure is due to an accelerated distribution of leukocytes to the tissues" It appears that their floor is of the same magnitude as our level below which leukocyte removal ceases This may contribute to the floor phenomenon It appears that the regulator of white cell removal is set to regulate to levels much below

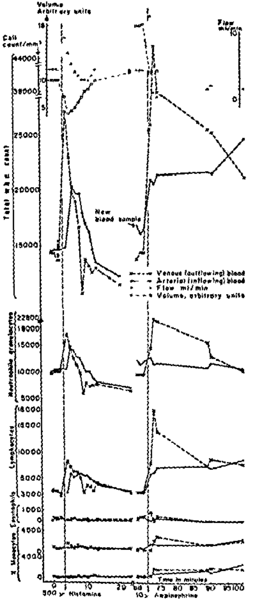


FIGURE 26 Effect of histamine and epinephrine on the regulation of leukocyte counts by the isolated dog spleen.

the normal range of circulating leukocyte counts Probably, the regulator of leukocyte production is set to regulate to levels higher than the normal circulating white cell count

The balance between these two regulators results in normal leukocyte levels and assures a continuous high rate of leukocyte production and elimination During elimination, leukocytes may perform useful "street-sweeping" functions As they travel up the tracheobronchial tree, granulocytes may phagocytize bacteria and foreign particulate matter and remove them through the

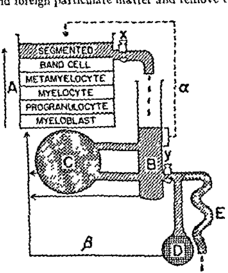


FIGURE 27 Schematic representation of the regulation of the granulocyte level Key *A*, bone marrow, *B*, peripheral blood, *C*, tissues, *D*, leukocyte destroying organs (liver, spleen), *E*, leukocyte-removing organs (lungs, intestine), α, effect of leukocyte level on release of cells from the bone marrow, β, effect of leukocyte decomposition products on bone marrow, *X*, regulation of cell production and release, and *Y*, regulation of cell elimination

gastrointestinal tract Lymphocytes passing through the intestinal wall may not only contribute to local antibacterial defense, but may also release antibodies when they disintegrate in the intestinal lumen, thus aiding in control of the bacterial flora Rapid rate of lymphocyte elimination and disintegration may contribute to continuous transport of lymphocytes and antibodies carried by them from the sites of antibody production to the periphery

FIGURE 27 schematically illustrates this hypothesis of double regulation of the leukocyte levels The symbol *X* represents the regulator of leukocyte production and release Leukocytes from the peripheral blood *B* can migrate into tissues *C*, and are removed by certain organs such as the liver and spleen *D* in which they disintegrate Disintegration products β (for example, the granulocyte expulsion factor of Steinberg, 1958) may play a role in regulating leukocyte production and release Leukocytes are removed continuously

through the respiratory and gastrointestinal tracts Y represents the regulator of leukocyte elimination The leukocyte level in the blood is determined by a balance between X and Y The effect of the peripheral leukocyte count on production and release from hemopoietic tissues is represented by α.

Gordon and associates (Kuna and Gordon, 1957, Dornfest and Gordon, 1958, Gordon *et al*, 1958) showed that leukocytes are released from the isolated rat hind limb when it is perfused with leukocyte-poor blood. Patt (1957) suggested that the granulocyte gradient may be an important factor in determining leukocyte release from the bone marrow. A high degree of leukocyte margination in the marrow capillaries may retard the release of new leukocytes

Summary

In cardiac catheterization experiments, disappearance of neutrophil granulocytes was observed in the pulmonary circulation of dogs

Catheterization of blood vessels leading into and out from various organs revealed continuous removal of all types of leukocytes in the hepatic and splenic circulation No leukocytes were removed in the capillary beds of extremities

Transfused, labeled leukocytes and leukocytes labeled in the blood stream with quinacrine disappeared from the circulation within 2 to 3 hours

Injection of epinephrine after the disappearance of transfused labeled cells resulted in the reappearance of labeled cells in the circulation. The main sources of leukocyte release were the pulmonary and splenic circulations and, to a lesser degree, the splanchnic circulation

Transfused, labeled leukocytes were removed chiefly by the lungs, liver, and spleen After several hours their number decreased in the lungs and spleen and increased in the bone marrow and lymphoid tissue (thymus).

Transfused, labeled granulocytes appeared to marginate to the capillary walls in the pulmonary circulation Some cells were released again into the blood stream Others slowly migrated through the alveolar walls into the alveoli, up the tracheobronchial tree, and appeared in the saliva, they were eventually swallowed and removed through the gastrointestinal tract

Transfused, labeled lymphocytes migrated through the walls of the small intestines into the intestinal lumen and were thus eliminated

It is suggested that during their elimination through the respiratory passages, granulocytes may aid in removing bacteria and particulate matter. During their intestinal elimination, lymphocytes may contribute to the prevention of infection from the gastrointestinal tract Disintegration in the intestines and release of antibodies may aid in controlling the intestinal flora

In the isolated heart-lung preparations of dogs, a regulatory function was detected Irrespective of the leukocyte count introduced into the preparation, the count was rapidly reduced to a level constant for each preparation and ranging from 500 to 1500 cells/cu mm Neutrophil granulocytes chiefly were affected by this regulation.

than that of the lungs No changes were seen in the leukocyte counts of blood circulated through isolated hind-limb preparations The leukocyte level-regulating function of lungs, liver, and spleen appears to be specific, not common to all capillary beds

Epinephrine and histamine caused contraction of the canine spleen and release of all types of leukocytes, including cells in various stages of degeneration Some evidence points toward decomposition of leukocytes in the liver and spleen

Although no leukocyte sequestration occurred in either the isolated or *in situ* hind limbs of dogs, epinephrine caused release of granulocytes There may be a balance between continuously marginating and demarginating cells in peripheral capillary beds. This balance may be disturbed by hemodynamic changes resulting in increased demargination Release of cells from the bone marrow may also be involved in this phenomenon

No difference was seen in the distribution of transfused, labeled, leukemic leukocytes between normal and spontaneously leukemic recipient mice No difference was seen in the intestinal and pulmonary removal of transfused labeled leukocytes between normal mice and mice with various transplanted, induced, and spontaneous leukemias

A double regulatory mechanism is proposed for the leukocyte level The elimination regulator regulates to levels much lower than the normal circulating leukocyte levels The production regulator regulates to leukocyte levels higher than normal By means of these two regulators, the leukocyte count is maintained within the normal range and a continuous high rate of leukocyte production and elimination is assured A continuous high rate of leukocyte removal through the respiratory and gastrointestinal passages protects these organs against infection and foreign particulates Continuous high rates of lymphocyte production, elimination through the gastrointestinal tract, and destruction in various organs may contribute to the transportation of a steady supply of antibodies from the sites of antibody formation to the periphery

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TISSUE CULTURE OF BONE MARROW

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With the possible exception of skin, bone marrow is the most easily obtainable, actively proliferating tissue in man. The fact that it gives rise to so many morphologically distinct elements of more or less well-defined functions and properties has constituted an irresistible challenge to the scientist to attempt its cultivation *in vitro*. In 1910 Carrel and Burrows[1] cultured bone marrow and in a few days observed the appearance of spindle-shaped cells that were later called fibroblasts. Generally, this term is applied to the spindle-shaped cells developing in cultures of blood-forming tissue, although some investigators challenge it on the grounds that the term should be reserved for cells derived from connective tissue. Since then the ubiquitous fibroblast has persisted to frustrate the attempts of many investigators to grow recognizable blood cells from marrow for more than a few days. A detailed review of the earlier work in this field appears in Bloom's article in Downey's *Handbook of Hematology*, published in 1938.[2]

Six years ago I ventured into this field, motivated by the conviction that if we knew what made blood cells differentiate, we should be in a favorable position to attack the leukemia problem. Since then various methods of growing marrow *in vitro* have been studied with the object of evaluating the potentialities and limitations of each as tools for the investigation of hematopoietic cells. I do not think that we have come much closer to the leukemia problem, but we have learned much about the behavior of blood cells *in vitro*, and we have had to revise many preconceived notions about growth and differentiation. The observations of many other workers have contributed greatly to our knowledge and philosophical approach to the problem, both the general tissue culture observations of Robert Chambers, C. G. Grand and Gladys Cameron, R. C. Parker, Margaret Sano, G. O. Gey, W. R. Earle and K. Sanford, C. M. Pomerat and Margaret Murray, and the more specifically hematopoietic observations of Harry Eagle, E. E. Osgood, Lawrence Berman, F. A. McCulloch, A. S. Gordon and John Biesele in United States and L. G. Lajtha and G. Astaldi abroad may be singled out for mention.

Early in our investigations my associates and I found that growth (in the sense of proliferation) and differentiation are mutually incompatible. When a blood cell has divided into two cells one may divide and one may differentiate, or both may do either, but neither can do both. It is hard to say exactly how far along the path of differentiation a cell may go and still be able to divide, but probably it is not very far. Micro-spectrophotometric determination of the desoxyribonucleic acid (DNA) content of the nuclei of maturing red cells showed a progressive decrease in the amount of DNA between the basophilic and the orthochromatic normoblast,[3] and it seems obvious that any division occurring after the beginning of this decrease of DNA would cause one or both of the daughter cells to end up with less than

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sional lines of cells will develop that appear morphologically similar to Earle's L strain. These cells, which he calls "altered cells of hemic origin," do not resemble any normal constituent of marrow very closely, although they have some of the characteristics of undifferentiated, reticuloendothelial cells. The significant thing about them is that they have established autonomy and can be subcultured readily in the same form. Osgood and Brooke[8] reported the recovery of several autonomous strains of monoblastic-appearing cells from marrow cultured for a long time in flat-sided bottles, and Berman *et al.*[9] have described the isolation in a similar type of preparation of quite a few strains of autonomous "epithelioid" cells developing from marrow. These cells are probably all closely related, if not of identical origin. They exhibit certain alterations from normal marrow cells in their chemical behavior and nutritional requirements and under the electron microscope certain morphologic alterations.* McCulloch's cells preserved antigenic properties of their tissue of origin, but had lost most of their ability to protect against radiation injury. While the establishment of these autonomous strains of cells is of the greatest interest and importance in itself, I consider it unwise to think of them as blood cells, since they have no recognized counterpart in human blood. They may prove to be extremely useful in the study of changes leading to the autonomy of malignant cells, with which they share several characteristics.[10] In fact, Billen[11] has recently reported that when MC32 cells obtained from McCulloch were injected intraperitoneally into irradiated mice of the same strain who were protected by simultaneous injections of homologous marrow cells, a number of the surviving recipients developed omental tumors.

One of the characteristics of fibroblastic cultures is the great amount of mitotic activity in process. During mitosis the cells draw in their pseudopods and assume a spheroidal shape, giving rise to two round cells that subsequently resume the spindle shape.

The source of fibroblasts has been in question ever since they were first described. Some observers considered them to be derived from the stroma of the marrow, but such a belief is not supported by the fact that they arise with equal rapidity from buffy coats of peripheral blood, marrow aspirates, and explants of marrow, spleen, or lymph nodes. In the days when the polyphyletists waged unremitting war on the monophyletists, the origin of the fibroblast was a hotly debated point. If we accept the multipotentiality of mesenchymal cells we must all be unitarians of a sort, and the polemics of yesteryear seem somewhat absurd, like the blind men's descriptions of the elephant. Several years ago we obtained from a patient with pernicious anemia a sample of marrow in which we could see the developing cells all the way back to the primary endothelium of the fatty marrow. As one progressed from the fatty marrow to the marrow sinusoid, the developing blood cells were seen to change from closely packed, flat, spindle-shaped cells with oval nuclei quite similar to fibroblasts to the typical round cells

* Osgood has subsequently established autonomous strains of all the different cell lines which, while not completely differentiated, do resemble more or less the cell types from which they are derived (see his article elsewhere in this publication).

the normal DNA complement In leukocytes the measurement is more difficult because of the shape of the nucleus, but changes of the same character as the cells mature appear probable On the other hand, in lymphocytes the nuclear DNA remains constant [4] These cells do not differentiate in the sense that the erythroid and myeloid elements do Following their release from the germinal centers, lymphocytes appear to go into a prolonged resting phase

The life span of the nucleated erythroblast is about five days; that of the leukocyte, about sixteen The lymphocyte's life span has been estimated as much longer, in fact, several months for leukemia lymphocytes [5] Pyknosis is a phenomenon characteristic of dying cells A corollary to these observations is the proposition that differentiated blood cells are dying cells, at least from a reproductive standpoint. We have coined the term mitotable to distinguish the younger cells still capable of mitosis from those that have matured beyond that point

We quickly learned to our satisfaction that an *in vitro* environment favorable for cell division was not favorable for cell differentiation, and vice versa The tissue culture techniques generally used for growing marrow are of two main types methods in which the marrow cells are suspended in a liquid medium, and those in which a marrow explant is fixed upon a sup

increasing signs of degeneration in the stained smear. Cell division take place in these cultures, most of it in the first 48 hours Our studies led u to the conclusion that those cells that enter the suspension in a mitotabl state will divide if conditions are favorable, but that there was little evidenc of a second generation of division Since normal marrow aspirates contai less than 5 per [illegible] unt when the aspir [illegible] galo blastic marrows [illegible] cell in the first 48 hours after suspension, which can be prevented by colchicin or A-Methopterin [6] The suspension type of culture has many advantage that make it ideal for short-term study of cell metabolism The cells ca be counted; they retain their identifying morphologic characteristics, th

not recur

In the fixed methods of marrow culture, differentiating cells that ente with the explant continue to mature and gradually disappear However within 48 hours, fibroblasts begin to appear and, in a fortnight, the entir culture may consist of fibroblasts, with a few round cells of an undifferen

in shallow Carrel flasks and are kept over a period of several months, occa

between that of normal and malignant tissue In certain instances, cells of normal origin have become definitely malignant following prolonged maintenance in culture [16]

We attempted to devise a technique that would grow fibroblasts in profusion under conditions that would facilitate their harvesting and resuspension In seeking for a method easier to use than the double cover-slip technique, or glass slivers in roller tubes, we adapted the method of Sano and Smith [17]

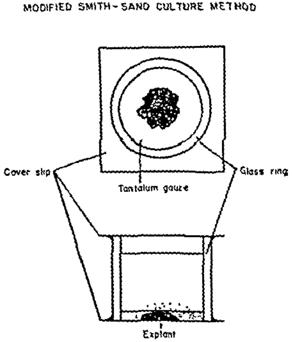


FIGURE 1 Diagram of the type of glass well used, showing the explant of marrow secured against the bottom cover slip by the tantalum gauze mesh

When I first saw the cultures in Sano's laboratory in 1948 I was struck by their simplicity but they never proved popular for general tissue culture use In this technique a well is created by sealing a glass cylinder on a cover slip The material to be cultured is placed in the well, medium is added, and the top is sealed with a second cover slip The growing cells can be observed directly under the microscope without disturbing the culture, medium can be changed or sampled by removing the top cover slip, if a permanent preparation is wanted the bottom cover slip with its adherent tissue is dropped into fixative, stained, and mounted This seemed to be an ideal method for the cultivation of fibroblasts that could then be harvested for resuspension

of the blood. The similarity in appearance of some fibroblastic cultures to some myelofibrotic marrows is striking. In studying our cultures to see which cells appeared to give rise to fibroblasts we found both reticulum cells and myelocytes that appeared
sively elongated into the spindl
similar to those in neutrophil
disappear after 4 to 6 weeks
suspended in a fluid medium, they round up and resemble primitive
cells of the hematopoietic system. We concluded that perhaps the fibro blast is the undifferentiated hemoblastic cell, structurally adapted to growing upon a surface

If the fibroblast developing in cultures of bone marrow is the undiffer entiated hematopoietic cell adapting itself to its environment in the absence of undefined specific stimuli to differentiation, several questions immediately arise If the fibroblast is reintroduced into an environment favorable fo hematopoietic differentiation, can it develop into a blood cell? In th absence of knowledge of the factors influencing such differentiation, a

has been established, the cells quickly tend to develop polyploidy,[14] afte which it is unlikely that they will revert to the original cell type of the paren tissue

If the fibroblast is the primitive stem cell of blood tissue, apparentl derived from the same cells that are supposed to repopulate the marro following radiation injury, does it still retain this potential? I have no

McCulloch's observations that his altered cells of hemic origin (whic were epithelioid rather than fibroblastlike in their appearance) had little o no protective effect [11]

Our own studies indicated that when the cultural environment that ha

demonstrated. It appears logical that, once an abnormal chromosom

be described may be obtained over a fairly wide range of concentrations of serous exudate and salt solution, and that the embryo extract was not essential, this extract was therefore soon discarded. Human plasma or serum may be used instead of malignant exudate. We now use 60 per cent exudate



FIGURE 3. Wright's stained concentrated suspension of cells from well culture at 28 days. Note presence of all elements including megakaryocytes.

and 40 per cent mixture 199 plus a drop of penicillin and Mycostatin. Similar results also can be obtained in Eagle's medium.

To our great surprise, no fibroblasts developed in these cultures. Instead, there was a continued outpouring of cells from the explant in gradually

Using wells of 18-mm. diameter and 10-mm depth on 22-mm. cover slips, we began with buffy coats of marrow aspirates and were disappointed to see only a few fibroblasts, none of which established themselves as a proliferating tissue. We then began to use whole marrow obtained from human ribs freshly resected in the operating room This would not adhere to the bottom cover slip, the fatty marrow always worked loose and floated up to the



FIGURE 2 Cells floating free around edges of explant after 16 days in culture

surface, where it dried out We heard Yoffey describe Trowell's[17a] meth of growing lymph node explants on a tantalum gauze mesh and we devise circle of tantalum gauze cut to fit the inside diameter of the well With t

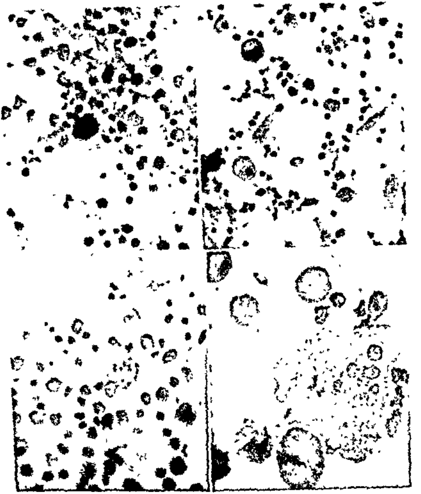


FIGURE 5 *Left top*, marrow grown 11 days at 8 mm depth *Right top* same marrow but grown 11 days at 4 mm *Left bottom* another marrow after 21 days at 4 mm The bottom cover slip has been completely taken over by macrophages fibroblasts, and multinucleated giant cells *Right bottom*, the same at a greater degree of magnification (all Wright s stain)

changes of medium, and optimal pH The examination of marrow growing

These never established themselves as true fibroblasts, but started to die

reticulum cells After several months, the number of cells produced after each weekly change of medium is small Examinations of the explant at this time reveal preservation of the marrow architecture with enough viable cells arising in the sinusoids to account for the continued delivery of cells into



FIGURE 4 Explant, paraffin block, H & E stain, 62 days

the surrounding medium These marrows resemble some hypoplastic marrows seen in patients (FIGURE 4).

This serendipitous discovery encouraged us to re-examine our concepts concerning marrow growth and differentiation If the fibroblast is the healthy, actively dividing cell, as we have intimated, it should thrive under favorable conditions of culture such as good oxygen exchange, frequent

Furthermore, when cultures started at the shallow depth had developed these cells and were then cultured at the regular depth, the macrophagelike *cells persisted but no new ones appeared, while the proportions of ordinary* hematopoietic cells increased in the culture (FIGURE 6) However, as long as the cultures contained the macrophagelike cells, they became acid more rapidly, although they were now being cultured at the full depth The *ability of the cultures to form the large cells* was *restricted to the initial* culture period, cultures that had been started at full depth and then changed to half-depth failed to develop them

Although the nature of these large cells is still obscure, it appears that the depth of the medium has something to do with the continued differentiation of hemic cells. Osgood defined the concept of what he called the gradient principle in marrow culture [18] He believed that the differentiation of different lines of blood cells is connected with the relation of the number of cells and the depth of the medium He calls the product of these two functions the gradient factor and lists optimal ranges of gradient factors for development of erythroid, myeloid, lymphoid, and plasmacytic elements In our cultures we have not observed any preferential line of differentiation for the first month, and we have not been able to discern any differences in the types of blood cells predominating at different depths on glass slides slanted at 45° in suspension cultures in French square bottles as described by Osgood We think that the depth factor is important probably because it decreases the available oxygen supply per cell Since the mitotic activity of a cell must require a large energy exchange, a poor oxygen supply might be expected to hinder such activity In short, metabolic conditions in our cultures are not sufficiently favorable to permit mitosis, therefore the cells must differentiate Enough cells divide in the depths of the marrow explant to keep the culture going but, since these cells differentiate, there is no way for one marrow explant to perpetuate itself, and its productivity gradually decreases over several months and eventually terminates from exhaustion of the primal elements

It is interesting to reflect on the fact that, although marrow in the body has a tremendous cellular potential, only a small fraction of the marrow cells capable of dividing do so This is just as well, *since otherwise we should all* have leukemia The oxygen quotient of marrow *in vitro* indicates a low level of oxidative respiratory activity [19] This is enhanced by acetate and other fatty acids, as well as pyruvate, indicating that both fat and carbohydrate may be utilized for energy That the *marrow normally may rely* more on fatty acids in its metabolism is suggested by the fact that its *in vitro* respiration is completely blocked by fluoroacetate, but only partially by malonic acid By using explants rich in fat (in which the marrow abounds) in these well cultures, which have limited aeration, we have succeeded perhaps in reproducing an environment more closely simulating that of marrow in the body

Having evolved fortuitously a technique that favors maturation of hematopoietic cells *along recognizable lines for an appreciable length of time*, we have turned to studying the effect of environmental alterations on differential

after a week However, in these shallow cultures, large round cells with

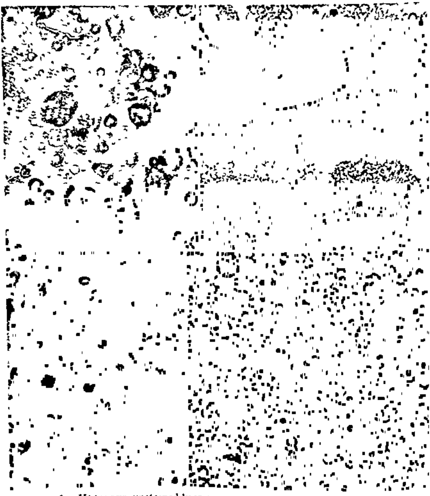


FIGURE 6 *Upper row*, unstained living preparation of marrow culture grown at 4 mm depth for 17 days, *lower row*, same culture after 3 days' incubation at 8 mm

failed to develop [illegible], fibroblastlike, or multinucleated cells associated with rapid acid formation

Furthermore, when cultures started at the shallow depth had developed these cells and were then cultured at the regular depth, the macrophagelike cells persisted but no new ones appeared, while the proportions of ordinary hematopoietic cells increased in the culture (FIGURE 6) However, as long as the cultures contained the macrophagelike cells, they became acid more rapidly, although they were now being cultured at the full depth The ability of the cultures to form the large cells was restricted to the initial culture period, cultures that had been started at full depth and then changed to half-depth failed to develop them

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after a week. However, in these shallow cultures, large round cells w large vesicular nuclei resembling tissue macrophages appeared. These w seen to divide and to develop into multinucleated, giant cells in some instar (FIGURES 5 and 6). Cultures containing these macrophagelike cells ap ently had a higher rate of metabolism as evidenced by a more rapid acidif



tion of the medium, necessitating changes every 2 to 3 days, in conti [illegible] deeper wells. The deeper well [illegible]

[illegible]

which [illegible] failed to develop the macrophagelike, [illegible]like, or multinucleate associated with rapid acid formation.

sions of cells harvested from the cultures, smeared, and stained with Wright's-Giemsa stain It can be seen that there was approximately twice as much erythropoiesis in the cultures grown with the active plasma filtrate as in the controls This confirms the observations of Gordon on erythropoiesis in the isolated perfused limb, and those of Matoth et al,[21] in using a 17-hour incubation of marrow suspended in a plasma clot

At present we are turning our attention to factors influencing the proliferation of myelocytes and lymphocytes in plasma and in extracts from patients with leukemia We believe that the capacity of the cell to divide is intrinsic, provided conditions are favorable for division, and that the direction in which the cell will differentiate is influenced by factors in its environment The leukemic state is one in which the ratio of dividing cells to differentiating cells is increased or even reversed Any tool that may shed light on the factors governing cell division or differentiation may lead us closer to the solution of this most challenging problem

Acknowledgment

These studies could not have been done without the help of the following assistants and technicians Harold T Swan, Morris Silverman, Frances Mottram, Alma Manieri, and Retha Odom

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growth It is not always easy to identify cells harvested from our wells after several weeks, but nucleated hemoglobin containing erythroblasts can usually be distinguished from other cells (even these may be confused easily with eosinophils in some preparations). Neither are the conditions of growth in our cultures yet so uniform that we can always compare events in one culture

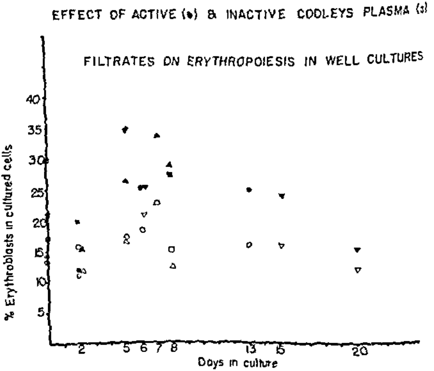


FIGURE 7 Effects of active erythropoietin containing boiled plasma filtrates (solid figures) and inactive filtrates (open figures) on erythropoiesis *in* bone marrow tissue cultures of the well type

material is added to the usual media, as previously described These filtrates* have been shown respectively to be active and inactive in their stimulation of erythropoiesis, both when injected *in vivo* and when used to

in vitro ... those cultures containing the active extract (FIGURE 7) The scattergram shows the results of six such experiments, the solid figures represent the percentage of recognizable red blood cell precursors *in* concentrated suspen

* Supplied by A S Gordon, New York University, New York, N Y

Part II. Hormone Control of Blood Cell Formation

THE ANEMIA OF HYPOPHYSECTOMIZED ANIMALS*

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University of Cincinnati College of Medicine, Cincinnati, Ohio

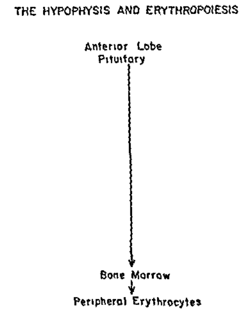


FIGURE 1 This figure and FIGURES 3, 5, 8, 10, 11, and 12 represent a gradual development of our hypothesis of how removal of the pituitary gland causes an anemia See FIGURE 2 and TABLE 1 for data

the study of anemia in hypophysectomized rats will give us more information concerning the fundamental process of red cell development, that is, erythropoiesis

Posthypophysectomy Anemia

FIGURE 1 shows, in diagrammatic form, that the anterior lobe of the pituitary has an effect on the bone marrow which in turn delivers erythrocytes

* The work reported in this paper was supported in part by Research Grant A-476 from the National Institute of Arthritis and Metabolic Diseases, Public Health Service, Bethesda, Md

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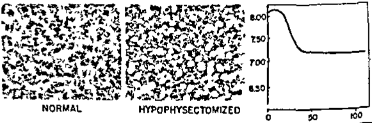
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TABLE 1
EFFECTS OF "ERYTHROPOIETIC HORMONE" ON THE BLOOD PICTURE OF HYPOPHYSECTOMIZED RATS
Treatment was started the day after surgery 50 µg per day for 30 days followed by 100 µg per day for 20 days ± = Standard Error

	Normal controls	Hypophysectomized No treatment		Hypophysectomized Erythropoietic hormone	
	8 Rats	5 Rats	Percentages of normal	5 Rats	Percentages of normal
Erythrocyte count in millions per cu mm	7 85 ±0 22	6 03 ±0 31	77	5 50 ±0 31	70
Hematocrit in per cent	45 2 ±1 4	31 8 ±1 2	70	27 0 ±0 9	60
Hemoglobin in gm per 100 cc	14 7 ±0 3	10 4 ±0 3	71	9 1 ±0 5	62
MCV in cu microns	57 5 ±0 7	52 7 ±1 2	92	49 5 ±1 8	86
MCH in micromicrograms	17 7 ±0 9	17 2 ±0 5	97	16 6 ±0 8	94
MCHC in per cent	32 5 ±0 5	32 6 ±0 3	100	33 6 ±1 1	103
Total white cell count in thousands per cu mm	14 8 ±1 3	20 8 ±2 2	141	24 0 ±4 0	162
Adrenal weights in mg	53 9 ±2 7	10 5 ±0 7	20	15 1 ±0 7	28
Thyroid weights in mg	12 4 ±0 5	6 9 ±0 8	56	9 8 ±0 6	79

while our data indicate that the Wistar and Sprague-Dawley strains show only 20* and 22 per cent decreases It should be noted that Berlin used the radioactive iron method of determining total cell volume, while we used the Evans blue method These data also indicate that the mean corpuscular hemoglobin is decreased after hypophysectomy, this is slight and due to the decrease in volume of the erythrocytes, since the mean corpuscular hemo-

to the peripheral blood Hypophysectomy is usually followed by a very slight rise in the erythrocyte count for 4 to 5 days. *This is followed by a* progressive decrease until approximately 40 days after hypophysectomy, no further decrease occurs and the blood picture plateaus for as long as a year the longest time interval we have studied. This is shown graphically in the upper right corner of FIGURE 2



	L E RATS			WISTAR RATS			S D RATS		
	Nor	Hyp	% Nor	Nor	Hyp	% Nor	Nor	Hyp	% Nor
TRC	8 17	5 79	71	8 52	7 10	83	8 33	7.38	89
Hematocrit	44 1	29.6	67	45 1	33 7	75	45 4	37 4	82
Hemoglobin	*14 7*	*9 8*	*67*	*15.9*	*12.1*	*76*	*15.4*	*12 6*	*83*
M C V	56 0	49 6	89	53 5	47 4	89	54 6	50.7	93
M C H	17 8	16.3	92	18 9	17 1	90	18 4	17 4	95
M C H C	31 9	32 9	103	35 3	35 9	102	33 3	34 2	101
Tot Cell Vol / 100 gm B W	—	—	—	2 53	2 0	80	2 37	1 85	78
Tot Marrow	1 78	1 32	74	1 85	1 59	86	1 87	1 41	75
Tot Erythroid	950	532	56	960	583	61	930	619	67
Tot Myeloid	627	603	96	690	769	*111*	739	596	*81*

Total cell volume 100 gm B W is expressed in cc, Tot Marrow, the total nucleated ce in millions/cu mm of marrow tissue, Tot Erythroid, the total erythroid elements, thousands/cu mm marrow tissue, and Tot Myeloid, the total myeloid elements, in th sands/cu mm marrow tissue

FIGURE 2 also presents a composite of all experiments we have done in t

hypophysectomy induces a 45 per cent decrease in circulating erythrocyt

THE HYPOPHYSIS AND ERYTHROPOIESIS

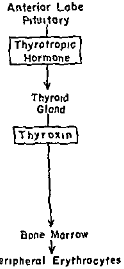


FIGURE 3 Tentative Hypothesis 1, possible role of hypothyroidism in posthypophysectomy anemia See FIGURE 4 for data



DAYS	60	60	50
T R C	7 13 (84)	7 97 (93)	7 80 (91)
Hematocrit	33 4 (73)	39 5 (86)	——
Hemoglobin	12 0 (73)	14.3 (87)	14 0 (85)
M C V	47 6 (88)	49 4 (91)	——
M C H	17 0 (88)	17 8 (93)	——
M C H C	35 5 (100)	35 9 (101)	——

FIGURE 4 Effects of thyroidectomy, hypophysectomy, and hypophysectomy followed by daily injections of 0 01 mg thyroxin on the blood and marrow conditions of the rat See FIGURE 2 for explanation of abbreviations used, numbers in parentheses represent percentages of normal.

globin concentration is normal in all animals Histologically all three strains exhibit a hypoplasia of the bone marrow after hypophysectomy; the number of nucleated cells per cu mm of bone marrow tissue is decreased in all three strains The number of erythroid elements per cu mm. of marrow decreased 44, 39, and 33 per cent in the 3 strains, respectively.

In summary, hypophysectomy induces an anemia of the slightly microcytic, hypochromic variety. This anemia is accompanied by a hypoplasia of the bone marrow in which there is a profound decrease in number of erythroid elements

Erythropoietic Hormone

Flaks *et al* [2] made an extract of beef pituitary glands and gave this orally to hypophysectomized animals They claimed erythropoiesis was stimulated and concluded that the pituitary secreted an erythropoietic hormone Contopoulos *et al* [3] have repeated this work and, although they could not find this erythropoietic factor in cattle pituitaries, they were able to do so in sheep glands They published two other papers[4, 5] describing how they fed this material to hypophysectomized rats, they claimed an alleviation of the anemia Since feeding pituitary extracts is open to some question, these workers followed this with an experiment in which they injected the material[6] As we had expressed some skepticism of this work, Contopoulos sent us some of their erythropoietic factor We injected this material into our hypophysectomized rats as directed and obtained negative results as can be seen in TABLE 1 It should be noted that we inject 3- to 4-month old female rats, while the California workers have been using rats approximately at weaning age

Thyroid

Since we do not believe the pituitary produces a specific erythropoietic hormone that affects the bone marrow directly, we are required to find an alternative explanation FIGURE 3 shows diagrammatically the possibility of the thyroid gland being involved in this picture. FIGURE 4 shows the effects on the blood picture of thyroid removal and thyroxin therapy in hypophysectomized animals Removal of the thyroid does induce an anemia, but one which is not as severe as that following hypophysectomy Treatment with thyroxin in doses of 0 01 mg per day restored the erythrocyte number to within 91 per cent of normal, the hemoglobin did not respond as well, being elevated to only 85 per cent of normal However, the thyroxin therapy must be considered effective when the above data are compared to those obtained in the hypophysectomized animals with no treatment. We shall have more details to present on the effects of thyroxin on posthypophysectomy anemia later in this paper. These findings would indicate that although the thyroid certainly does have an influence on erythropoiesis hypothyroidism alone cannot account for posthypophysectomy anemia.

Adrenal Cortex

The adrenal gland should not be forgotten (FIGURES 5 and 6). Adrenalectomized rats also exhibit an anemia, but this has been found to be of a te

THE HYPOPHYSIS AND ERYTHROPOIESIS

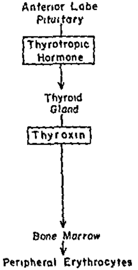
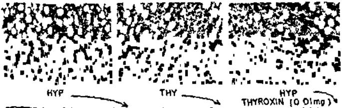


FIGURE 3 Tentative Hypothesis 1, possible role of hypothyroidism in posthypophysectomy anemia. See FIGURE 4 for data



DAYS	60	60	50
T R C	7 13 (84)	7 97 (93)	7 80 (91)
Hematocrit	33 4 (73)	39 5 (86)	——
Hemoglobin	12 0 (73)	14 3 (87)	14 0 (85)
M C V	47 6 (88)	49 4 (91)	——
M C H	17 0 (88)	17 8 (93)	——
M C H C	35 5 (100)	35 9 (101)	——

FIGURE 4 Effects of thyroidectomy, hypophysectomy, and hypophysectomy followed by daily injections of 0 01 mg thyroxin on the blood and marrow conditions of the rat See FIGURE 2 for explanation of abbreviations used, numbers in parentheses represent percentages of normal

porary nature,[7-10] due according to Fisher,[10] to the presence of adı remnants. Injections of 0 6 mg. cortisone acetate daily[11] do have an e on the blood picture of hypophysectomized rats but do not return it to a lutely normal figures The peripheral blood was altered to a degree brought the hematocrit and hemoglobin to within 95 and 99 per cent of mal, but the total cell volume per 100 gm. body weight remained at a figure of 73 per cent of normal The bone marrow showed an even gr

THE HYPOPHYSIS AND ERYTHROPOIESIS

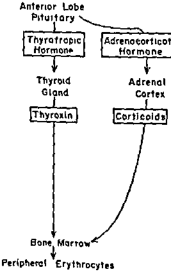


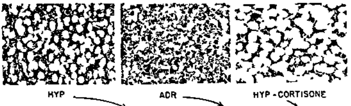
FIGURE 5 Tentative Hypotheses 2 and 3, the possible role of hypoadrenocortic (see FIGURE 6 for data) or combined hypothyroidism and hypoadrenocorticalism FIGURE 7 for data) in posthypophysectomy anemia

hypoplasia than is found after hypophysectomy and no treatment. C sone acetate therapy seems to stimulate delivery of erythrocytes to peripheral blood, but at the expense of the bone marrow; obviously, the l marrow cannot keep up with the demand

Thyroid and Adrenal

Combined thyroidectomy and adrenalectomy (FIGURE 7) do indu peripheral anemia very similar to that induced by hypophysectomy.

27 per cent above normal after the dual operation. In addition, although the number of erythroid elements was decreased following thyroidectomy and adrenalectomy, this decrease was not as severe as that found following hypophysectomy. FIGURE 7 also shows the effects of treating hypophysectomized rats with 0.005 mg. of thyroxin and 0.6 mg. of cortisone daily,[11, 14]

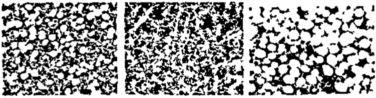


DAY	60	60	50
TRC	7.13 (84)	7.50 (88)	7.36 (89)
Hematocrit	33.4 (73)	40.0 (87)	41.9 (95)
Hemoglobin	12.0 (73)	13.4 (82)	14.3 (99)
Retic	—	—	1.8 (257)
MCV	47.6 (88)	49.4 (91)	56.9 (107)
MCH	17.0 (88)	17.8 (93)	19.4 (110)
MCHC	35.5 (100)	35.9 (101)	34.1 (103)
Tot. Cell Vol. / 100 gm. B.W.	—	—	1.85 (73)
Tot. Marrow	—	—	1.094 (56)
Tot. Erythroid	—	—	629.3 (62)
Tot. Myeloid	—	—	363.8 (48)

FIGURE 6. Effects of adrenalectomy, hypophysectomy, and hypophysectomy followed by daily injections of 0.6 mg. cortisone acetate on the blood and marrow pictures of the rat. See FIGURE 2 for explanation of abbreviations used. Numbers in parentheses represent percentages of normal.

the peripheral anemia of hypophysectomized animals [illegible]

addition, both cell types, erythroid and myeloid, were decreased in the bone marrow of these animals to 83 and 52 per cent of normal. Therefore, a combination of thyroidectomy and adrenalectomy, although producing a peripheral anemia similar to that of hypophysectomy, cannot be said to duplicate the anemia in all respects. Cortisone and thyroxin therapy, although alleviating the peripheral anemia, has done so at the expense of the bone marrow.



DAY	60	60	50
TRC	7 15 (84)	6 65 (76)	8 44 (102)
Hematocrit	32 0 (71)	35 6 (79)	46 2 (104)
Hemoglobin	10 6 (69)	11 8 (77)	15 5 (105)
Retic	—	—	2.5 (357)
MCV	44 8 (83)	53 9 (99)	54 7 (103)
MCH	14 9 (80)	17 9 (96)	18 3 (104)
MCHC	33 3 (96)	33 3 (96)	33 5 (101)
Tot Cell Vol / 100 gm BW	—	—	2 60 (103)
Tot Marrow	1 47 (84)	2 22 (127)	1 360 (69)
Tot Erythroid	486 7 (53)	653 1 (72)	830 9 (83)
Tot Myeloid	719 8 (115)	1160 1 (186)	392 0 (52)

FIGURE 7 Effects of combined thyroidectomy and adrenalectomy, hypophysectomy, and hypophysectomy followed by daily injections of 0 005 mg thyroxin and 0 6 mg cortisone acetate on the blood and marrow conditions of rats See FIGURE 2 for explanation of abbreviations, numbers in parentheses represent percentages of normal

Growth Hormone

Another factor lost to the hypophysectomized rat is growth hormone (FIGURE 8) Fruhman *et al* [15] and we[16] have shown that the bone marrow in hypophysectomized rats is stimulated by growth hormone injections (FIGURE 9). The nucleated cells per cu mm. of marrow tissue were 28 per cent above normal, and the erythroid elements were actually counted as 49 per cent above normal In spite of these findings, growth hormone injections do not eliminate the peripheral anemia This has been found to be true by several groups of workers [15-21] The total cell volume per 100 gm body [illegible] of normal Fruhman *et al* [15] and later [illegible] the lack of effect on peripheral blood [illegible] with this therapy, but we agree [illegible]

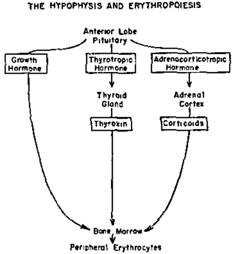


FIGURE 8 Tentative hypotheses 4 and 5, the possible role of loss of growth hormone or loss of growth hormone combined with hypothyroidism and hypoadrenocorticalism in posthypophysectomy anemia See FIGURE 9 for data

Growth Hormone + Thyroid + Adrenal

alleviated every aspect of posthypophysectomy anemia except for the total erythroid elements in the bone marrow We feel that this exception is simply a matter of dosage Therefore, in summary, the anemia following removal of the hypophysis would seem to be due to the lack of thyroid and adrenocortical activity and to loss of growth hormone found in such animals

Oxygen Need

The diagram in FIGURE 8 indicates that these hormones are producing their effect on erythropoiesis by affecting the bone marrow directly We believe that there is no such direct effect of these products and glands on the bone marrow, but that they influence it secondarily through their effects on general metabolism, this in turn affects the oxygen need of the animal and, therefore, the rate of erythropoiesis This concept is diagramed in FIGURE 10 What evidence do we have for making this hypothesis?

The nature of posthypophysectomy anemia must be considered Although there is an anemia in these animals, the bone marrow can function in the absence of the pituitary gland This can be shown in several ways

(1) The erythrocyte count and the hematocrit and hemoglobin levels in hypophysectomized rats do not show a continued decrease, as mentioned previously, approximately 40 days after hypophysectomy the blood picture ceases to change and levels off, the number of erythrocytes fluctuating at approximately the same level for as long as a year after hypophysectomy.



DAYS	125	50	90
TRC	6 90 (86)	6 67 (83)	9 22 (111)
Hematocrit	38 6 (86)	37 1 (83)	47 0 (103)
Hemoglobin	13 4 (86)	12 4 (79)	16 0 (103)
Retic	0 3 (100)	2 1 (600)	1 3 (118)
M C V	55 9 (101)	55 6 (100)	51 0 (93)
M C H	19 4 (101)	18 5 (96)	17 3 (93)
M C H C	34 7 (99)	33 4 (96)	34 0 (100)
Tot Cell Vol / 100 gm B W	1 68 (74)	1 41 (62)	2 39 (103)
Tot Marrow	1309 (74)	2 268 (128)	1724 (99)
Tot Erythroid	670 2 (74)	1356 3 (149)	709 1 (82)
Tot Myeloid	472 6 (72)	687 2 (105)	784 4 (112)

FIGURE 9 Effects of hypophysectomy, hypophysectomy followed by daily injec (gradually increasing doses of 0 2 mg to 0 8 mg) of growth hormone, and hypophysec followed by daily injections of 0 005 mg thyroxin, 0 6 mg cortisone acetate and 0 2 n 0 8 mg growth hormone on the blood and marrow conditions of the rat See FIGURE explanation of abbreviations used Numbers in parentheses represent percentag normal

(2) Although Meyer *et al* [17] could not induce responses in the bone ma of hypophysectomized rats by decreasing the oxygen tension, Feigin Gordon[24] were able to do so by subjecting the animals to a more s decrease

(3) Silberglest[25] and Finkelstein *et al* [26] found that the hypophysector rat responded to hemorrhage by an erythropoiesis, the blood returning level typical of hypophysectomized rats only

(4) Baker *et al.*[27] have shown that the hypophysectomized rat tr with total irradiation will become more anemic than untreated hypoph tomized rats, but will recover to such an extent that the blood pictu typical of the untreated hypophysectomized animals.

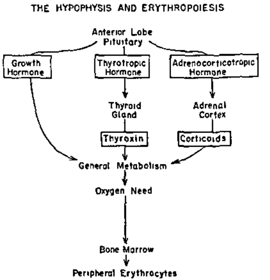


FIGURE 10 Tentative hypothesis 6, the possible mediation of hormone loss via lowered general metabolism and subsequent decreased oxygen need on the part of the animal See TABLES 2, 3, and 4 for data

TABLE 2
BLOOD PICTURE AND OXYGEN CONSUMPTION IN HYPOPHYSECTOMIZED ADULT FEMALE RATS BEFORE AND AFTER A BLOOD TRANSFUSION
Average amount of blood transfused was 4 5 ml ± = Standard Error

	Normal controls	Hypophysectomized before transfusion		Hypophysectomized after transfusion	
	13 Rats	7 Rats	Percentages of normal	7 Rats	Percentages of normal
Erythrocyte count in millions per cu mm	8 21 ±0 16	6 99 ±0 17	86	9 39 ±0 36	114
Hematocrit in per cent	46 3 ±0 75	36 3 ±1 10	79	48 1 ±1 60	103
Hemoglobin in gm per 100 cc	14 9 ±0 23	13 2 ±0 28	89	16 3 ±0 46	109
Oxygen consumption in liters per square meter body surface per hour	7 79 ±0 22	5 26 ±0 17	68	4 71 ±0 24	61

(5) In addition, the remarkable response of the hypophysectomized rat to cobalt should not be forgotten.[21, 22] Therefore, the hypophysectomized rat has marrow tissue that will function properly in the absence of the pituitary gland

TABLE 3
PERIPHERAL BLOOD AND BONE MARROW PICTURES AND OXYGEN CONSUMPTION IN 5 GROUPS OF RATS

(1) [illegible]
5([illegible]

0 05 mg, (5) same as group 3, but with the daily dose of thyroxin increased to 0 10 mg ± = Standard Error

	Normal controls	Hyp no treatment		Hyp Cor + G H + 0 01 mg Thy /d × 40		Hyp Cor + G H + 0 05 mg Thy /d × 40		Hyp Cor + G H + 0 10 mg Thy /d × 40	
	10 Rats	5 Rats	Percentages of normal	9 Rats	Percentages of normal	7 Rats	Percentages of normal	6 Rats	Percentages of normal
Erythrocyte count in millions per cu mm	8 50 ±1 37	7 89 ±0 41	92	9 42 ±0 14	110	9 56 ±0 29	112	9 98 ±0 30	117
Hematocrit in per cent	46 0 ±0 69	38 2 ±1 20	83	47 9 ±0 46	104	52 3 ±1 80	113	52 3 ±0 90	113
Hemoglobin in gm per 100 cc	15 5 ±0 18	13 5 ±0 51	87	15 2 ±0 26	98	16 4 ±0 38	105	17 2 ±0 45	110
Total red cell volume in cc per 100 gm B W	2 52 ±0 06	2 15 ±0 15	85	2 99 ±0 12	118	3 78 ±0 06	150	4 18 ±0 17	165
Oxygen consumption in liters per square meter body surface per hour	9 48 ±0 34	6 27 ±0 13	66	9 43 ±0 28	99	12 87 ±0 43	135	15 04 ±0 54	158
Total nucleated cells in millions per cu mm marrow tissue	1 95 ±0 08	1 46 ±0 11	75	2 30 ±0 09	118	2 62 ±0 10	134	2 67 ±0 13	137
Total no erythroid elements in thousands per cu mm marrow tissue	888 3 ±36	653 3 ±33	73	1262 1 ±97	142	1578 7 ±142	177	1690 8 ±142	190
Total no myeloid elements in thousands per cu mm marrow tissue	947 7 ±30	710 5 ±80	74	911 4 ±66	96	905 5 ±59	95	861 5 ±146	90

the above data presented

brought back to normal by means of transfusions, the oxygen consumption is still only 61 per cent of normal [33]

Another experiment[34] was conducted to watch changes in the blood and marrow pictures brought about by a previously successful therapy in hypophysectomized rats of thyroxin, cortisone, and growth hormone, but with increases in thyroxin dosage included to alter oxygen consumption Hypophysectomized rats were treated with 0 6 mg cortisone acetate, gradually increasing doses of growth hormone (0 2 to 0 8 mg), and 0 01, 0 05 or 0 10 mg of thyroxin per day The data are presented in TABLE 3 It can be seen

TABLE 4

TOTAL ERYTHROCYTE VOLUME AND OXYGEN CONSUMPTION IN 5 GROUPS OF RATS

(1) [illegible] for [illegible] is [illegible]

	Normal controls	Hyp no treatment		Hyp thyroxin 0 01 mg /day		Hyp thyroxin 0 05 mg /day		Hyp thyroxin 0 10 mg /day	
	32 Rats	23 Rats	Percentages of normal	14 Rats	Percentages of normal	16 Rats	Percentages of normal	8 Pats	Percentages of normal
Body weight in grams	267 ±2 80	213 ±3 73	80	191 ±2 50	72	163 ±2 57	61	163 ±4 8	61
Total erythrocyte volume in cc / 100 gm B W	2 56 ±0 06	2 04 ±0 04	80	2 13 ±0 05	83	3 11 ±0 10	121	2 74 ±0 11	107
Oxygen Consumption in liters per square meter body surface per hour	9 01 ±0 14	6 06 ±0 20	67	9 02 ±0 25	100	11 17 ±0 20	124	9 61 ±0 36	107

readily that an increase in the dose of thyroxin increased the total red cell volume per 100 gm body weight, and concomitant increases of a similar nature were found in oxygen consumption Incidentally, this is the first therapy we have tried in hypophysectomized rats (with the exception of cobalt)[28, 29] where a real polycythemia has been produced

If oxygen need is the only factor involved in posthypophysectomy anemia, why did we obtain rather discouraging results with thyroidectomy and [illegible]

marked decreases in weight, all the animals were treated until the blood picture was normal, that is, 45 days at 0 01-mg , 25 days at 0 05-mg , and 10 days at 0 10-mg dose levels Once again, in spite of the rather drastic

effects of this treatment in hypophysectomized rats, the blood picture a[illegible] oxygen consumption were affected in a similar manner.

Since better results were obtained when cortisone and growth hormo[illegible] were combined with thyroxin than when thyroxin was used alone, it might [illegible] assumed that this indicated that cortisone and growth hormone were affect[illegible] the bone marrow directly and that oxygen need was not the entire explan[illegible] tion. This may be true, but if the data are examined carefully it can be se[illegible] that a combination of thyroxin, cortisone, and growth hormone has a mo[illegible] profound influence on oxygen consumption than thyroxin alone. When

THE HYPOPHYSIS AND ERYTHROPOIESIS

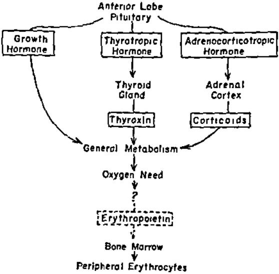


FIGURE 11 Tentative hypothesis 7, erythropoietin as a possible intermediary betwee[illegible] oxygen need and the bone marrow

[illegible]

directly on the marrow, the combined treatment has a more profound influence on oxygen consumption

Erythropoietin

If a decreased oxygen need is the explanation for the anemia found in [illegible] animals this still does not ans[illegible] the question of how [illegible] dicated in FIGURE 11, [illegible] be the intermediary

between oxygen need and the bone marrow, we are investigating this point at the present time

Summary

FIGURE 12 is presented as a summary. We believe that hypophysectomy induces an anemia by the resultant hypothyroidism, hypoadrenocorticalism, and lack of growth hormone that occur after such surgery We also hypothesize that these losses do not exert themselves directly upon the bone marrow but, secondarily, through a decrease in general metabolism, this decrease in

THE HYPOPHYSIS AND ERYTHROPOIESIS

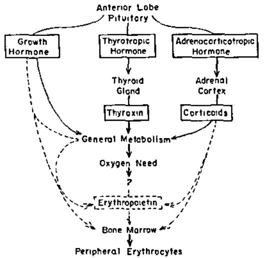


FIGURE 12 Tentative hypothesis 8, a summary of our present interpretation of the manner in which the anterior lobe of the pituitary gland affects erythropoiesis

metabolism, in turn, decreases the animal's need for oxygen This decreased oxygen need results in a decreased erythropoiesis in the bone marrow, possibly through a decreased amount of erythropoietin in the plasma The extra dotted lines running from growth hormone to erythropoietin or bone marrow directly or by means of general metabolism have been added because we know that growth hormone can influence the bone marrow without raising oxygen consumption, it is also difficult to separate a phenomenon such as

feel that these dotted lines are necessary until these points of confusion are resolved

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INFLUENCE OF HYPOXIC STIMULI UPON BLOOD FORMATION IN ENDOCRINE-DEFICIENT ANIMALS*

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Gordon *et al* [1] have demonstrated that hypoxia, the generally acknowledged "fundamental erythrocytogenic stimulus," results in morphological and physiological alterations in various endocrine glands, including the pituitary, adrenal, thyroid, and gonads. The suggestion has been made[2] that hypoxic stimuli may affect hematopoiesis as a result of operating through hormonal mechanisms Along these lines, the experiments of Stewart *et al.*[3] revealed that the hypophysectomized rat does not display an erythropoietic response to lowered barometric pressures corresponding to a simulated altitude of 16,000 feet However, while Feigin and Gordon[4] have confirmed these findings, of especial significance are their results that demonstrate that the hypophysectomized rat can respond with increased erythropoiesis to an extent similar to that experienced by intact controls when exposed to low pressures

poietic stress imposed by lowered barometric pressures of 310 8 mm Hg[5] In view of the above findings, and since the possibility exists that more than one hormone produced by the endocrine glands is the fundamental stimulus for erythrocytosis, a detailed correlation of the peripheral blood changes to the bone marrow cytology in pleuriglandular-deficient animals subjected to lowered barometric pressures was made.

Materials and Methods

Two hundred and ten young adult female rats of the Sprague-Dawley strain, weighing 135 to 170 gm at the time of hypophysectomy or sham

mental period Data include records of only completely operated animals and animals surviving the entire experimental period

Peripheral blood from the tail was employed for all procedures. Red and

† Supplied by

white cell counts were made in duplicate, the former were required to agree to within ±4 per cent and the latter to within ±10 per cent Differential counts were made from smears treated with Wright's stain Eosinophils were estimated by the method of Randolph[6] Reticulocyte counts were made from dry smears of peripheral blood stained with new methylene blue[7] One thousand erythrocytes were counted and the reticulocytes expressed as a percentage of these Hemoglobin concentrations were determined by the acid hematin method with a photoelectric colorimeter Duplicate hematocrit determinations from heparinized blood, spun at 12,000 rpm for 7 min, were made by a capillary micromethod and were required to agree to within ±1 mm. Duplicate serial blood volume determinations were made by a modification of the method of Wang and Hegsted[8] Animals were lightly anesthetized with ether and the front of the neck shaved and washed with alcohol By blunt dissection the jugular vein was exposed and injected with 0 3 ml of Evans blue (T1824), which contains 0 6 mg of the dye Eight min later, 0 5 ml of blood was withdrawn by cardiac puncture into a syringe moistened with a heparin solution Two tenths ml of the heparinized blood was added to 5 8 ml of 0 9 per cent sodium chloride, mixed gently, and then centrifuged at 3,000 rpm for 30 min The color of the supernatant solution was measured against a blank, using plasma diluted 29 times The photometric reading was made at 610 mμ and compared with a standard of Evans blue (2 3 μg/ml in 0 9 per cent sodium chloride) Plasma volume was calculated, employing the formula

$$\text{Plasma volume} = \frac{\text{Blood volume} \times \text{per cent plasma (100}-\text{hematocrit)}}{100}$$

Control hematological determinations were carried out in all animals before experimental treatment was begun The experimental groups were established as follows

Experiment 1 hypophysectomized and control animals following 21 days of exposure Twenty hypophysectomized and 10 sham-hypophysectomized control animals, operated 10 weeks previously, were subjected to lowered barometric pressures in a specially constructed low-pressure chamber The animals received 4-hour exposures to pressures of 310 8 mm Hg (equivalent to an altitude of 20,000 feet) every second day for 21 days Hematological examinations were made at weekly intervals

Experiment 2 adrenalectomized-hypophysectomized and adrenalectomized control animals following 21 days of exposure Thirty hypophysectomized and 20 sham-hypophysectomized control animals, operated 10 weeks previously, were adrenalectomized and examined at weekly intervals for 21 days After 21 days of adrenalectomy, 15 adrenalectomized-hypophysectomized and 10 adrenalectomized-sham-hypophysectomized rats were subjected to the same experimental procedures as in Experiment 1

Experiment 3 castrate-hypophysectomized and castrate control animals Thirty hypophysectomized and sham-hypophysectomized control animals, operated 10 weeks previously, were castrated and examined at weekly inter-

vals for 21 days Twenty-one days after castration, 15 castrate-hypophysectomized and 10 castrate-sham-hypophysectomized rats were subjected to the same experimental procedures as in Experiment 1.

Experiment 4 castrate-adrenalectomized-hypophysectomized and castrate adrenalectomized control animals. Thirty hypophysectomized and 20 sham hypophysectomized control animals, operated 10 weeks previously, were castrated and adrenalectomized in a single operative procedure and examined at weekly intervals for 21 days. Twenty-one days after castration plus adrenalectomy, 15 castrate-adrenalectomized-hypophysectomized and 10 castrate-sham-hypophysectomized rats were subjected to the same experimental procedures as in Experiment 1

At the termination of the observation periods, all rats were anesthetized lightly with ether and exsanguinated by cardiac puncture. The left femur was dissected, cracked open, and the

down gently in the serum with a glass pipette Smears were made and fixed in absolute methanol for 2 min and treated with May-Grunwald stain Smears treated by Ralph's technique[9] were also prepared. For each animal, 1000 nucleated cells were counted for determination of the myelograms

addition, pituitary, adrenal glands, spleen, thymus, and heart tissues were fixed in 10 per cent formalin for histological analysis. To date our histological studies are incomplete, our findings will be reported in a subsequent publication

Weekly body weight measurements were made for all animals

RESULTS*

Experiment 1

Peripheral blood. The effects of 72 days of hypophysectomy are shown in TABLES 1, 2, and 3 It will be noted (TABLE 1) that this operation results in

part, the anemia of hypophysectomy in this study is of the normocytic, normochromic variety A microcytosis is evident only at 14 and 21 days after hypophysectomy when compared to sham-operated animals examined at this time FIGURE 8 reveals that the total blood volumes of untreated

significant

in plasma volume levels noted after hypophysectomy are not statistically significant

TABLE 3 indicates that statistically significant increases in total white cell counts occur by the sixth week through the tenth week following hypophysectomy The differential count showed a tendency to neutrophilia and lymphopenia No significant alterations in the peripheral total and differential

TABLE 1
EFFECTS OF HYPOPHYSECTOMY UPON ERYTHROCYTIC VALUES
(Means ± Standard Errors)

	No of days postop	No of rats	RBC million/ (cu mm)	Hemoglobin gm (percentages)	Reticulocyte (percentages)	Hematocrit (percentages)
Hypx	7	20	8 79 ± 0 15	16 66 ± 0 45	2 4 ± 0 14	47 7 ± 1 7
	14	20	8 83 ± 0 22	15 90 ± 0 49	2 3 ± 0 20	47 9 ± 1 2
	21	20	8 64 ± 0 34	15 64 ± 0 55	1 0 ± 0 23	46 5 ± 1 2
	28	20	8 35 ± 0 31	15 89 ± 0 39	0 8 ± 0 10	45 8 ± 1 5
	35	20	7 91 ± 0 27	14 53 ± 0 60	1 1 ± 0 20	44 4 ± 1 7
	42	20	7 63 ± 0 24	14 36 ± 0 81	0 9 ± 0 19	42 5 ± 1 2
	51	20	7 63 ± 0 25	14 02 ± 0 44	1 3 ± 0 40	42 9 ± 1 7
	58	20	7 31 ± 0 14	14 66 ± 0 35	1 3 ± 0 45	39 5 ± 1 5
	65	20	7 16 ± 0 31	14 73 ± 0 44	0 8 ± 0 26	42 4 ± 1 4
	72	110	7 04 ± 0 23	14 32 ± 0 42	0 7 ± 0 29	40 1 ± 1 5
Controls	7	10	8 25 ± 0 23	15 77 ± 0 54	1 8 ± 0 24	46 4 ± 1 5
	14	10	7 90 ± 0 14	15 57 ± 0 45	2 2 ± 0 22	47 0 ± 1 6
	21	10	7 94 ± 0 43	15 51 ± 0 39	1 9 ± 0 28	46 8 ± 1 2
	28	10	8 10 ± 0 30	16 03 ± 0 61	2 0 ± 0 40	45 7 ± 1 7
	35	10	7 97 ± 0 34	15 74 ± 0 61	2 3 ± 0 30	46 7 ± 1 9
	42	10	8 05 ± 0 25	16 18 ± 0 38	1 9 ± 0 38	47 0 ± 1 6
	51	10	7 86 ± 0 11	16 41 ± 0 42	1 3 ± 0 45	46 8 ± 1 4
	58	10	8 29 ± 0 23	16 62 ± 0 51	1 5 ± 0 27	47 9 ± 1 2
	65	10	8 36 ± 0 19	16 70 ± 0 54	1 8 ± 0 28	48 4 ± 1 0
	72	80	8 50 ± 0 23	16 32 ± 0 44	2 0 ± 0 19	48 5 ± 1 5

white cell counts are induced by sham-operated animals Eosinophil counts reveal significant elevations only in the hypophysectomized group

FIGURE 1 and TABLES 2 and 5 indicate the effects of 21 days of lowered barometric pressure upon the peripheral blood picture of hypophysectomized and control rats The application of lowered barometric pressures results in significant increases in red cell, hemoglobin, hematocrit, and reticulocyte determinations that are similar in the hypophysectomized and in the control group of animals (FIGURE 1) Mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration values are unaltered in both groups of animals after exposure to the stimulus of lowered barometric pressures (TABLE 2) Total blood volume levels of exposed hypophysectomized and sham-operated rats undergo progressive increases accompanied by no significant alterations in plasma volume values

TABLE 5 reveals that decreases in total white cell counts occur in the hypophysectomized animals subjected to the lowered barometric pressures Total white cell counts in sham-operated rats are unaffected by the reduced

TABLE 2
EFFECTS OF LOW PRESSURE UPON SOME ERYTHROCYTIC VALUES OF SHAM-HYPOPHYSECTOMIZED AND HYPOPHYSECTOMIZED RATS
(Means ± Standard Errors)

	No of rats	Mean corp vol (cu μ)	Mean corp hb ($\mu\mu$g)	Mean corp hb conc percentages
Hypx				
Postop 7 days	20	54 3 ± 0 9	18 9 ± 0 3	34 9 ± 0 8
14 days	20	54 2 ± 0 7	18 0 ± 0 8	33 2 ± 0 3
21 days	20	53 8 ± 0 6	18 1 ± 0 4	33 6 ± 1 0
28 days	20	54 9 ± 0 8	19 0 ± 0 6	34 7 ± 0 7
35 days	20	56 1 ± 1 2	18 4 ± 0 8	32 7 ± 0 2
42 days	20	55 7 ± 1 3	18 8 ± 0 5	33 8 ± 0 5
51 days	20	56 1 ± 1 5	18 3 ± 0 9	32 7 ± 0 4
58 days	20	54 0 ± 1 3	20 0 ± 0 8	37 1 ± 0 9
65 days	20	59 2 ± 1 4	20 6 ± 0 3	34 7 ± 0 6
72 days	130	57 0 ± 0 8	20 3 ± 0 5	35 7 ± 1 0
Controls				
Postop 7 days	10	56 3 ± 1 0	19 1 ± 0 6	34 0 ± 0 9
14 days	10	59 5 ± 0 9	19 7 ± 0 6	33 1 ± 0 5
21 days	10	58 7 ± 1 0	19 5 ± 0 7	33 1 ± 0 8
28 days	10	56 4 ± 0 7	19 8 ± 0 4	35 1 ± 0 6
35 days	10	58 6 ± 0 9	19 8 ± 0 5	33 7 ± 0 9
42 days	10	58 4 ± 0 9	20 1 ± 0 8	34 4 ± 1 0
51 days	10	59 5 ± 1 0	20 9 ± 0 9	35 1 ± 0 8
58 days	10	57 8 ± 1 2	20 1 ± 0 6	34 7 ± 1 0
65 days	10	57 9 ± 0 9	20 0 ± 0 4	34 5 ± 0 7
72 days	80	57 1 ± 1 4	19 2 ± 0 7	33 7 ± 0 9
Hypx (72 days postop)				
Exposed 7 days	20	56 4 ± 1 1	19 3 ± 0 3	34 2 ± 0 7
14 days	20	55 0 ± 1 5	19 7 ± 0 5	35 8 ± 1 0
21 days	20	54 7 ± 1 2	19 0 ± 0 4	34 8 ± 0 8
Controls (72 days postop)				
Exposed 7 days	10	56 3 ± 0 9	19 3 ± 0 6	34 3 ± 0 6
14 days	10	59 6 ± 1 5	19 6 ± 0 4	32 9 ± 1 1
21 days	10	58 9 ± 1 4	20 2 ± 0 7	34 3 ± 0 7

pressure Likewise, differential white cell determinations show no con-

Bone marrow studies The myelograms of the hypophysectomized rats (TABLE 13) reveal significant decreases in the percentages of nucleated erythroid elements after 72 days of operation, accompanied by a significant elevation in the concentration of lymphocytes and a tendency to rise in the numbers of young neutrophilic forms

TABLE 13 reveals similar changes in the marrow cellular components of

TABLE 3
EFFECTS OF HYPOPHYSECTOMY UPON WHITE CELL COUNTS
(Means ± Standard Errors)

	No of days postop	No of rats	Total WBC thousand, cu mm	Eosinophils, cu mm	Lymphocytes, percentages	Neutrophils, percentages
Hypx	7	20	16 3 ± 1 5	415 1 ± 18 0	87 0 ± 2 5	12 0 ± 2 5
	14	20	16 8 ± 1 4	332 4 ± 16 9	84 3 ± 3 8	13 7 ± 3 4
	21	20	13 5 ± 1 7	385 6 ± 22 0	85 1 ± 3 0	13 1 ± 2 3
	28	20	14 9 ± 1 9	301 8 ± 25 1	83 9 ± 2 7	14 6 ± 5 4
	35	20	18 3 ± 1 9	297 5 ± 12 7	80 5 ± 2 9	17 6 ± 2 8
	42	20	20 3 ± 2 4	318 9 ± 13 5	81 8 ± 3 6	15 6 ± 2 2
	51	20	19 1 ± 2 3	312 9 ± 15 2	83 4 ± 2 7	15 1 ± 3 0
	58	20	19 5 ± 2 5	351 3 ± 14 7	79 8 ± 2 2	18 5 ± 3 3
	65	20	24 8 ± 2 8	254 5 ± 17 2	80 6 ± 3 9	17 9 ± 4 2
	72	130	22 4 ± 1 9	344 9 ± 16 7	78 5 ± 2 1	20 8 ± 3 0
Controls	7	10	16 2 ± 1 2	151 0 ± 16 0	82 0 ± 2 7	16 4 ± 2 9
	14	10	16 5 ± 1 7	193 2 ± 17 9	80 7 ± 2 2	17 6 ± 4 3
	21	10	16 7 ± 2 5	139 9 ± 24 3	83 8 ± 3 5	14 6 ± 3 2
	28	10	18 4 ± 2 8	180 8 ± 18 0	81 1 ± 3 7	13 9 ± 4 4
	35	10	15 3 ± 1 9	131 0 ± 21 0	88 0 ± 1 4	11 3 ± 4 5
	42	10	16 9 ± 1 8	209 8 ± 37 0	86 9 ± 4 1	10 9 ± 2 3
	51	10	13 3 ± 1 1	198 7 ± 17 2	85 8 ± 2 1	12 2 ± 2 2
	58	10	13 6 ± 2 3	212 0 ± 20 0	88 3 ± 4 3	10 1 ± 3 0
	65	10	14 9 ± 2 3	193 8 ± 18 0	84 4 ± 3 4	13 8 ± 3 1
	72	80	14 6 ± 2 4	185 0 ± 17 4	83 9 ± 2 9	14 0 ± 4 9

the control and hypophysectomized rats subjected to the lowered barometric pressures

Organ weights TABLE 14 indicates that hypophysectomy decreases significantly the spleen and adrenal weights of the female rat The low-pressure stimulus employed is of sufficient intensity to increase significantly the adrenal weights with a tendency to decrease the thymus weights in the control animals Pituitary gland weights were not affected by this treatment On the other hand, hypophysectomized-exposed rats reveal significant decreases in the weights of spleen, thymus, and adrenal glands when compared to control-exposed animals However, the hypophysectomized-exposed group reveals no changes from the unexposed-hypophysectomized animals, except for a significant decrease in thymus weights in the exposed group

Experiment 2

Peripheral blood FIGURE 2 and TABLES 4 and 7 indicate the effects of adrenalectomy upon the peripheral blood picture of hypophysectomized and sham-hypophysectomized rats FIGURE 2 reveals significant decreases in red cell, hemoglobin, and hematocrit values in both groups of animals after 21 days following adrenalectomy. A significant rise in reticulocyte values

TABLE 4
EFFECTS OF LOW PRESSURE UPON SOME ERYTHROCYTIC VALUES OF ADRENALECTOMIZED, RATS

		No of rats	Mean corp. vol (cu μ)	Mean corp hb (μμg)	Mean corp hb conc, percentages
Hypx. (72 days postop) + Adx postop		130	57 0 ± 0 8	20 3 ± 0 5	35 7 ± 1 0
	7 days	30	59 9 ± 1 2	20 8 ± 0 8	34 9 ± 0 9
	14 days	30	59 5 ± 0 3	20 6 ± 0 5	34 6 ± 0 9
	21 days	30	57 0 ± 0 6	20 5 ± 0 7	35 9 ± 0 4
Controls (72 days postop) + Adx postop		80	57 1 ± 1 4	19 2 ± 0 7	33 7 ± 0 9
	7 days	20	57 5 ± 0 8	19 4 ± 0 5	33 7 ± 0 4
	14 days	20	60 8 ± 0 3	20 8 ± 0 2	34 2 ± 0 6
	21 days	20	55 2 ± 1 0	19 2 ± 0 6	34 8 ± 1 1
Hypx (72 days postop) + Adx (21 days postop) Exposed	7 days	15	56 1 ± 1 3	20 3 ± 0 4	36 1 ± 0 8
	14 days	15	63 6 ± 1 1	20 0 ± 0 6	31 3 ± 0 7
	21 days	15	59 3 ± 0 5	19 2 ± 0 7	32 4 ± 0 3
Controls (72 days postop) + Adx (21 days postop) Exposed	7 days	10	59 6 ± 0 8	20 0 ± 0 2	33 4 ± 0 9
	14 days	10	61 0 ± 0 6	20 1 ± 0 5	33 0 ± 0 9
	21 days	10	60 3 ± 0 5	20 4 ± 0 5	33 9 ± 0 6

occurs in adrenalectomized control rats at the 21-day postoperative period, whereas a significant elevation is observed only in the 7-day adrenalectomized- [illegible]

in sham-operated rats, significant increases in mean corpuscular hemoglobin values are noted. FIGURE 8 reveals that the total blood volume of sham-hypophysectomized animals undergoes progressive decreases following adrenal ablation Plasma volume levels are unaffected No further sig-

nificant alterations in blood-volume levels occur in the hypophysectomized group following adrenalectomy

Total white cell counts in both groups (TABLE 7) are unaffected by adrenalectomy Differential white cell determinations show a neutropenia and lymphocytosis in adrenalectomized-hypophysectomized animals Of interest is the depression in eosinophil values observed in adrenalectomized-hypophysectomized rats, in contrast to the elevation noted in the adrenalectomized-control group

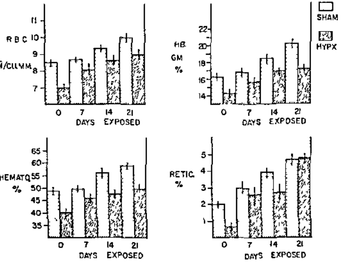


FIGURE 1 Effects of low pressure upon erythrocytic values of hypophysectomized and sham-hypophysectomized rats Vertical lines through the bars represent ± 1 standard error of the mean

The effects of 21 days of lowered barometric pressure in adrenalectomized-hypophysectomized and adrenalectomized control rats are shown in FIGURE 3 and TABLES 4 and 8 Significant increases in the peripheral red blood cell parameters examined are noted in both groups of animals (FIGURE 3) Exposure to lowered barometric pressures results in significant rises in the mean corpuscular volume levels 14 and 21 days after adrenalectomy In addition, at this time only the adrenalectomized-hypophysectomized group displays decreases in mean corpuscular hemoglobin concentrations (TABLE 4) Significant increases in total blood volume occur in both groups of adrenalectomized rats following the stimulus of lowered barometric pressures However, plasma-volume levels remain unaltered (FIGURE 8)

Total white cell, eosinophile, and differential white cell counts show no consistent differences in both groups of exposed animals compared to the unexposed groups of rats (TABLE 8)

Bone marrow studies TABLE 13 reveals that adrenalectomized-hypophysectomized animals possess significant decreases in immature and mature

Experiment 2

Peripheral blood FIGURE 2 and TABLES 4 and 7 indicate the effects of adrenalectomy upon the peripheral blood picture of hypophysectomized and sham-hypophysectomized rats. FIGURE 2 reveals significant decreases in red cell, hemoglobin, and hematocrit values in both groups of animals after 21 days following adrenalectomy A significant rise in reticulocyte values

TABLE 4
EFFECTS OF LOW PRESSURE UPON SOME ERYTHROCYTIC VALUES OF ADRENALECTOMIZED, HYPOPHYSECTOMIZED AND ADRENALECTOMIZED, SHAM-HYPOPHYSECTOMIZED RATS
(Means ± Standard Errors)

		No of rats	Mean corp vol (cu μ)	Mean corp hb (μμg)	Mean corp hb conc, percentages
Hypx (72 days postop) +		130	57 0 ± 0 8	20 3 ± 0 5	35 7 ± 1 0
Adx postop	7 days	30	59 9 ± 1 2	20 8 ± 0 8	34 9 ± 0 9
	14 days	30	59 5 ± 0 3	20 6 ± 0 5	34 6 ± 0 9
	21 days	30	57 0 ± 0 6	20 5 ± 0 7	35 9 ± 0 4
Controls (72 days postop) +		80	57 1 ± 1 4	19 2 ± 0 7	33 7 ± 0 9
Adx postop	7 days	20	57 5 ± 0 8	19 4 ± 0 5	33 7 ± 0 4
	14 days	20	60 8 ± 0 3	20 8 ± 0 2	34 2 ± 0 6
	21 days	20	55 2 ± 1 0	19 2 ± 0 6	34 8 ± 1 1
Hypx (72 days postop) + Adx (21 days postop) Exposed					
	7 days	15	56 1 ± 1 3	20 3 ± 0 4	36 1 ± 0 8
	14 days	15	63 6 ± 1 1	20 0 ± 0 6	31 3 ± 0 7
	21 days	15	59 3 ± 0 5	19 2 ± 0 7	32 4 ± 0 3
Controls (72 days postop) + Adx (21 days postop) Exposed					
	7 days	10	59 6 ± 0 8	20 0 ± 0 2	33 4 ± 0 9
	14 days	10	61 0 ± 0 6	20 1 ± 0 5	33 0 ± 0 9
	21 days	10	60 3 ± 0 5	20 4 ± 0 5	33 9 ± 0 6

[illegible] control rats at the 21 [illegible]

[illegible]

mals at the 14-day [illegible] weeks following adrenalectomy in sham-operated rats, significant increases in mean corpuscular hemoglobin values are noted. FIGURE 8 reveals that the total blood volume of sham-hypophysectomized animals undergoes progressive decreases following adrenal ablation Plasma volume levels are unaffected. No further sig-

TABLE 6

EFFECTS OF LOW PRESSURE UPON SOME ERYTHROCYTIC VALUES OF CASTRATE-HYPOPHYSECTOMIZED, CASTRATE SHAM HYPOPHYSECTOMIZED, CASTRATE-ADRENALECTOMIZED HYPOPHYSECTOMIZED, AND CASTRATE-ADRENALECTOMIZED SHAM HYPOPHYSECTOMIZED RATS
(Means ± Standard Errors)

	No of rats	Mean corp vol (cu μ)	Mean corp hb (μμg)	Mean corp hb conc, percentages
Hypx (72 days postop) + castration	130	57.0 ± 0.8	20.3 ± 0.5	35.7 ± 1.0
postop 7 days	30	60.8 ± 1.3	20.1 ± 0.6	33.1 ± 0.9
14 days	30	58.2 ± 0.9	19.5 ± 0.5	33.6 ± 1.0
21 days	30	58.3 ± 0.8	19.2 ± 0.7	32.9 ± 1.3
Controls (72 days postop) + castration	80	57.1 ± 1.4	19.2 ± 0.7	33.7 ± 0.9
postop 7 days	20	59.8 ± 1.1	20.0 ± 1.0	33.5 ± 0.4
14 days	20	60.6 ± 1.3	19.8 ± 0.8	32.7 ± 0.8
21 days	20	58.6 ± 0.9	20.2 ± 1.0	34.5 ± 0.7
Hypx (72 days postop) + castration (21 days postop)				
Exposed 7 days	15	55.0 ± 1.3	19.8 ± 0.5	36.0 ± 1.0
14 days	15	55.7 ± 1.1	18.6 ± 0.7	33.3 ± 0.7
21 days	15	54.5 ± 0.7	18.7 ± 0.5	34.4 ± 0.8
Controls (72 days postop) + castration (21 days postop)				
Exposed 7 days	10	59.9 ± 0.9	20.0 ± 0.8	33.5 ± 0.9
14 days	10	62.3 ± 1.4	21.2 ± 0.9	34.1 ± 1.1
21 days	10	59.5 ± 0.6	21.3 ± 0.8	35.8 ± 1.0
Hypx (72 days postop) + cast + Adx				
postop 7 days	30	58.3 ± 1.0	20.8 ± 0.7	35.7 ± 1.2
14 days	30	54.6 ± 0.4	20.1 ± 0.9	36.8 ± 1.1
21 days	30	53.0 ± 0.7	19.4 ± 0.3	36.7 ± 0.7
Controls (72 days postop) + cast +				
Adx postop 7 days	20	58.8 ± 0.9	21.6 ± 1.2	32.5 ± 1.2
14 days	20	59.1 ± 0.5	19.8 ± 0.2	33.0 ± 1.3
21 days	20	60.8 ± 0.6	21.1 ± 0.9	35.0 ± 0.9
Hypx (72 days postop) + cast + Adx (21 days postop)				
Exposed 7 days	15	54.7 ± 0.9	19.7 ± 0.5	35.9 ± 1.4
14 days	15	59.2 ± 0.8	19.6 ± 0.8	33.1 ± 0.5
21 days	15	58.9 ± 0.7	19.4 ± 0.6	32.9 ± 0.7
Controls (72 days postop) + cast + Adx (21 days postop)				
Exposed 7 days	10	62.8 ± 1.2	20.1 ± 1.0	31.9 ± 1.4
14 days	10	68.2 ± 0.7	20.7 ± 0.8	30.3 ± 0.6
21 days	10	59.3 ± 0.7	21.2 ± 0.7	35.7 ± 1.3

neutrophilic forms, a lowering in eosinophile percentages, and significant elevations in lymphocytic forms in the bone marrow, as compared to adrenalectomized-control animals The rise in lymphocyte percentages is significantly higher than the elevation observed in hypophysectomized rats The myelograms of adrenalectomized-controls reveal significant increases in lymphocyte, young, and mature neutrophile percentages in comparison to unoperated control animals

The marrow cellular components of adrenalectomized-hypophysectomized and adrenalectomized control rats subjected to lowered barometric pressures

TABLE 5

EFFECTS OF LOW PRESSURE UPON WHITE CELL COUNTS OF HYPOPHYSECTOMIZED AND SHAM-HYPOPHYSECTOMIZED RATS

(Means ± Standard Errors)

	No of rats	Total WBC thousand cu mm	Eosinophils cu mm	Lymphocytes percentages	Neutrophils percentages
Hypx (72 days postop)	130	22 4 ± 1 9	344 9 ± 16 7	78 5 ± 2 1	20 8 ± 3 0
Exposed 7 days	20	15 2 ± 2 4	148 9 ± 16 0	83 0 ± 4 1	15 4 ± 4 1
14 days	20	15 5 ± 1 9	249 4 ± 11 9	82 3 ± 5 0	15 5 ± 4 0
21 days	20	15 1 ± 2 7	325 8 ± 17 1	82 3 ± 4 0	16 0 ± 4 7
Controls (72 days postop)	80	14 6 ± 2 4	185 0 ± 17 4	83 9 ± 2 9	14 0 ± 4 9
Exposed 7 days	10	14 1 ± 1 7	377 1 ± 20 0	82 2 ± 3 0	15 0 ± 1 7
14 days	10	13 8 ± 2 1	294 5 ± 21 4	82 9 ± 2 7	13 3 ± 2 0
21 days	10	14 0 ± 1 8	359 9 ± 17 5	82 6 ± 3 0	15 0 ± 2 0

reveal similar erythropoietic responses and decreases in lymphocytic forms In addition, a decrease in the mature neutrophilic percentages is noted in adrenalectomized controls

Organ weights Although no changes are observed in the organ weights of adrenalectomized control rats, significant decreases in the spleen weights of adrenalectomized-hypophysectomized animals are seen (TABLE 14) The stimulus of lowered barometric pressures imposes increases in the weights of spleen and thymus tissue only in the adrenalectomized controls. Significant decreases in spleen and thymus weights are noted in exposed adrenalectomized-hypophysectomized animals when compared to exposed adrenalectomized controls

Experiment 3

Peripheral blood The effects of castration upon the peripheral blood of hypophysectomized and sham-hypophysectomized rats are shown in FIGURE 4 and TABLES 6 and 9 Except for decreases in eosinophil levels in the hypophysectomized animals, castration in both groups results in no further significant changes in the peripheral blood parameters examined

Lowered barometric pressures in both groups evoke erythropoietic responses

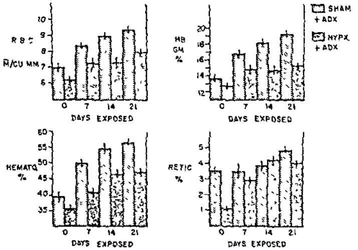


Figure 3 Effects of low pressure upon erythrocytic values of adrenalectomized-sham hypophysectomized and adrenalectomized hypophysectomized rats Vertical lines through the bars represent ± 1 standard error of the mean

Bone marrow studies Table 13 reveals no significant changes in castrate controls However, increases in lymphocytic forms and decreases in nucleated erythrocytes are evident in castrate-hypophysectomized rats in comparison to castrate control animals Similar erythropoietic and myeloid changes are noted in both groups when exposed to lowered barometric pressures

Table 8
Effects of Low Pressure upon White Cell Counts of Adrenalectomized Hypophysectomized and Adrenalectomized-Sham-Hypophysectomized Rats
(Means ± Standard Errors)

	No of rats	Total WBC thousand, cu mm	Eosinophils, cu mm	Lymphocytes, percentages	Neutrophils, percentages
Hypx (72 days postop) +					
Adx (21 days postop)	30	17 6 ± 1 9	158 2 ± 16 7	87 4 ± 2 0	10 1 ± 3 1
Exposed 7 days	15	16 6 ± 2 6	190 9 ± 15 2	87 6 ± 2 1	11 2 ± 2 7
14 days	15	17 8 ± 1 7	146 5 ± 12 5	91 7 ± 3 1	7 5 ± 2 3
21 days	15	15 6 ± 1 3	164 5 ± 13 4	92 3 ± 3 0	7 5 ± 3 5
Controls (72 days postop) +					
Adx (21 days postop)	20	14 9 ± 1 8	479 0 ± 17 4	80 5 ± 3 5	14 0 ± 3 0
Exposed 7 days	10	13 8 ± 1 9	388 4 ± 27 4	81 2 ± 4 0	14 0 ± 1 9
14 days	10	12 9 ± 2 0	244 1 ± 30 4	84 0 ± 2 5	12 2 ± 2 3
21 days	10	14 0 ± 1 7	342 1 ± 18 4	82 4 ± 3 3	15 1 ± 2 4

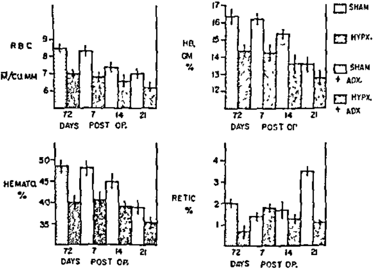


FIGURE 2 Effects of adrenalectomy upon erythrocytic values of hypophysectomized and sham-hypophysectomized rats Vertical lines through the bars represent ± 1 standard error of the mean

(FIGURE 5) accompanied by significant increases in total blood volume concentrations (FIGURE 9) A comparison of exposed hypophysectomized-castrate and exposed-castrate-sham-hypophysectomized animals (TABLE 6) indicates that the mean corpuscular volume levels display decreases in the hypophysectomized group No significant changes are evident in the white series (TABLE 10), except for significant reductions in eosinophil counts at the 21-day period in castrate-hypophysectomized rats

TABLE 7
EFFECTS OF ADRENALECTOMY UPON WHITE CELL COUNTS OF HYPOPHYSECTOMIZED AND SHAM HYPOPHYSECTOMIZED RATS
(Means ± standard errors)

	No of rats	Total WBC thousand, cu mm	Eosinophils, cu mm	Lymphocytes, percentages	Neutrophils, percentages
Hypx (72 days postop) +	130	22 4 ± 1 9	344 9 ± 16 7	78 5 ± 2 1	20 8 ± 3 0
Adx postop 7 days	30	19 7 ± 2 9	240 2 ± 14 2	87 1 ± 4 1	12 2 ± 2 6
14 days	30	17 9 ± 1 5	259 9 ± 12 9	88 0 ± 3 7	12 0 ± 2 8
21 days	30	17 6 ± 1 0	158 2 ± 16 7	87 4 ± 2 0	10 1 ± 3 1
Controls (72 days postop)	80	14 6 ± 2 4	185 0 ± 17 4	83 9 ± 2 9	14 0 ± 4 9
+ Adx postop 7 days	20	13 8 ± 1 6	201 8 ± 16 4	84 0 ± 2 4	12 4 ± 4 0
14 days	20	13 0 ± 2 1	503 4 ± 28 4	84 1 ± 4 4	17 1 ± 5 0
21 days	20	14 9 ± 1 8	479 0 ± 17 1	80 5 ± 3 5	14 0 ± 3 0

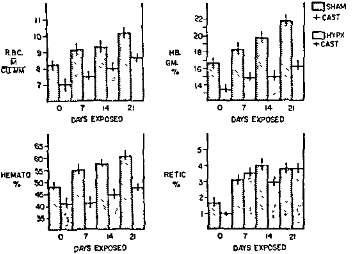


FIGURE 5 Effects of low pressure upon erythrocytic values of castrate-hypophysectomized and castrate sham-hypophysectomized rats Vertical lines through the bars represent ±1 standard error of the mean

TABLE 10
EFFECTS OF LOW PRESSURE ON WHITE CELL COUNTS OF CASTRATE HYPOPHYSECTOMIZED AND CASTRATE-SHAM-HYPOPHYSECTOMIZED RATS
(Means ± Standard Errors)

		No of rats	Total WBC thousand, cu mm	Eosinophils, cu mm	Lymphocytes percentages	Neutrophils, percentages
Hypx (72 days postop) + castration (21 days postop)		30	19.1 ± 1.4	183.2 ± 15.0	82.7 ± 2.9	16.3 ± 3.0
Exposed	7 days	15	19.5 ± 3.0	127.7 ± 17.6	84.5 ± 2.7	14.7 ± 4.0
	14 days	15	18.0 ± 2.7	114.1 ± 12.7	78.6 ± 3.6	20.0 ± 4.1
	21 days	15	21.4 ± 1.5	95.5 ± 12.5	79.2 ± 3.2	20.2 ± 3.4
Controls (72 days postop) + castration (21 days postop)		20	16.0 ± 2.3	162.8 ± 11.7	82.4 ± 2.7	15.3 ± 4.0
Exposed	7 days	10	16.2 ± 2.2	183.2 ± 22.4	83.7 ± 3.3	15.5 ± 3.1
	14 days	10	18.6 ± 1.9	194.2 ± 24.0	78.7 ± 2.1	20.8 ± 2.9
	21 days	10	15.1 ± 2.4	183.3 ± 15.8	78.7 ± 4.6	19.0 ± 4.2

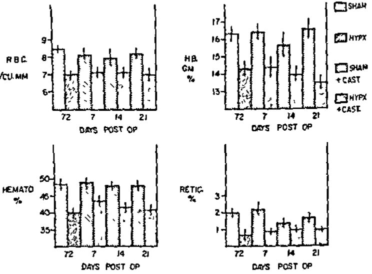


FIGURE 4 Effects of castration upon erythrocytic values of hypophysectomized and sham-hypophysectomized rats Vertical lines through the bars represent ±1 standard error of the mean

Organ weights TABLE 14 indicates no significant changes in organ weights of castrate controls, whereas castrate-hypophysectomized animals reveal significant decreases in spleen and adrenal weights. The reductions are of the same magnitude as untreated hypophysectomized animals. Lowered barometric pressures evoke elevations in adrenal weights, with reductions in thymus weights in castrate-control rats On the other hand, exposed groups

TABLE 9
EFFECTS OF CASTRATION UPON WHITE CELL COUNTS OF HYPOPHYSECTOMIZED AND SHAM HYPOPHYSECTOMIZED RATS
(Means ± Standard Errors)

	No of rats	Total WBC thousand, cu mm	Eosinophils, cu mm	Lymphocytes, percentages	Neutrophils, percentages
Hypx (72 days postop) +	130	22.4 ± 1.9	344.9 ± 16.7	78.5 ± 2.1	20.8 ± 3.0
castration postop 7 days	30	17.3 ± 1.7	182.6 ± 19.2	82.1 ± 3.9	16.6 ± 4.1
14 days	30	22.7 ± 1.9	168.3 ± 12.4	85.6 ± 5.1	13.5 ± 2.7
21 days	30	19.1 ± 1.4	183.2 ± 15.0	82.7 ± 2.9	16.3 ± 3.0
Controls (72 days postop) +	80	14.6 ± 2.4	185.0 ± 17.4	83.9 ± 2.9	14.0 ± 4.9
castration postop 7 days	20	18.1 ± 1.7	208.0 ± 14.6	87.1 ± 4.0	11.4 ± 4.4
14 days	20	16.7 ± 2.1	138.8 ± 28.0	86.0 ± 3.1	12.8 ± 4.7
21 days	20	16.0 ± 2.3	162.8 ± 11.7	82.4 ± 2.7	15.3 ± 4.0

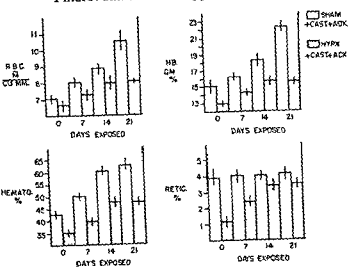


FIGURE 7 Effects of low pressure upon erythrocytic values of castrate-adrenalectomized-hypophysectomized and castrate adrenalectomized-sham hypophysectomized rats Vertical lines through the bars represent ±1 standard error of the mean

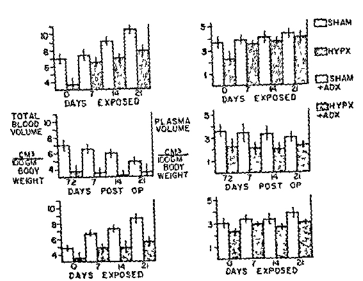


FIGURE 8 Effects of low pressure upon the blood volume changes of hypophysectomized, sham hypophysectomized, adrenalectomized-hypophysectomized, and adrenalectomized sham hypophysectomized rats Vertical lines through the bars represent ±1 standard error of the mean

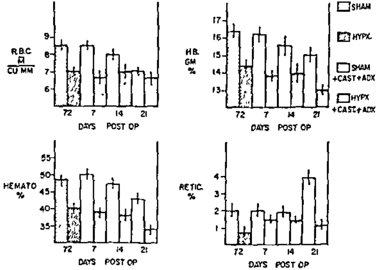


FIGURE 6 Effects of castration plus adrenalectomy upon erythrocytic values of hypophysectomized and sham-hypophysectomized rats Vertical lines through the bars represent ±1 standard error of the mean

TABLE 11
EFFECTS OF CASTRATION + ADRENALECTOMY UPON WHITE CELL COUNTS OF HYPOPHYSECTOMIZED AND SHAM-HYPOPHYSECTOMIZED RATS
(Means ± Standard Errors)

		No of rats	Total WBC thousand, cu mm	Eosinophils, cu mm	Lymphocytes, percentages	Neutrophils, percentages
Hypx (72 days postop) + Cast + Adx, postop		130	22.4 ± 1.9	344.9 ± 16.7	78.5 ± 2.1	20.8 ± 3.0
	7 days	30	20.4 ± 1.6	386.6 ± 15.3	85.0 ± 2.2	13.8 ± 3.5
	14 days	30	17.5 ± 1.8	230.0 ± 17.5	86.2 ± 2.7	13.1 ± 3.8
	21 days	30	13.1 ± 2.1	223.4 ± 15.2	84.6 ± 3.3	13.7 ± 4.0
Controls (72 days postop) + Cast + Adx postop		80	14.6 ± 2.4	185.0 ± 17.4	83.9 ± 2.9	14.0 ± 4.9
	7 days	20	16.3 ± 1.4	368.8 ± 18.9	77.2 ± 2.5	21.1 ± 2.3
	14 days	20	19.3 ± 1.2	397.9 ± 17.0	81.1 ± 2.3	17.4 ± 2.8
	21 days	20	15.8 ± 1.7	305.3 ± 19.1	84.1 ± 2.7	14.6 ± 3.4

group is depressed significantly as to total white cell and eosinophil counts in comparison to the control group. When compared to the castrate-hypophysectomized and castrate control animals, the addition of adrenalectomy results in elevations in the eosinophil values of both groups studied (TABLE 11)

Twenty-one days of lowered barometric pressures in castrate-adrenalectomized-hypophysectomized and castrate-adrenalectomized control rats (FIGURE 7) indicate increased erythrocytogenic responses in both groups

TABLE 12

EFFECTS OF LOW PRESSURE ON WHITE CELL COUNTS OF CASTRATE ADRENALECTOMIZED HYPOPHYSECTOMIZED AND CASTRATE ADRENALECTOMIZED-SHAM HYPOPHYSECTOMIZED RATS

(Means ± Standard Errors)

	No of rats	Total WBC thousand, cu mm	Eosinophils, cu mm	Lymphocytes percentages	Neutrophils, percentages
Hypx (72 days postop) + Cast + Adx (21 days postop)	30	13.1 ± 2.1	223.4 ± 15.2	84.6 ± 3.3	13.7 ± 4.0
Exposed 7 days	15	14.2 ± 2.3	161.3 ± 15.1	85.0 ± 2.9	13.4 ± 2.5
14 days	15	13.5 ± 2.0	160.2 ± 20.0	83.7 ± 3.0	14.6 ± 3.9
21 days	15	17.5 ± 1.7	134.8 ± 18.4	82.3 ± 3.1	16.0 ± 2.9
Controls (72 days postop) + Cast + Adx (21 days postop)	20	15.8 ± 1.7	305.3 ± 19.1	84.1 ± 2.7	14.6 ± 8.4
Exposed 7 days	10	16.8 ± 2.2	364.2 ± 20.3	82.1 ± 3.0	16.1 ± 2.7
14 days	10	15.1 ± 2.7	212.5 ± 18.1	75.1 ± 2.5	22.4 ± 4.1
21 days	10	15.7 ± 1.9	379.8 ± 20.0	74.1 ± 4.0	23.3 ± 2.5

TABLE 6 indicates that the mean corpuscular volume displays significant rises accompanied by decreases in mean corpuscular hemoglobin concentrations in exposed castrate-adrenalectomized-hypophysectomized rats Similar alterations are noted only at the 14-day postexposure period in the control animals FIGURE 9 reveals that significant increases in the total volume levels are produced by lowered barometric pressures after adrenal and ovarian removal in both groups No accompanying changes in plasma volume values are induced by the stimulus employed

Eosinophil determinations after 21 days of exposure were affected in the white blood cell parameters examined (TABLE 12), revealing significant depressions in the hypophysectomized group in contrast to the elevations noted in the control animals These changes are significant when compared to each other and to the unexposed postoperative series The observed decreases in lymphocyte numbers accompanied by increases in neutrophil percentages are significant

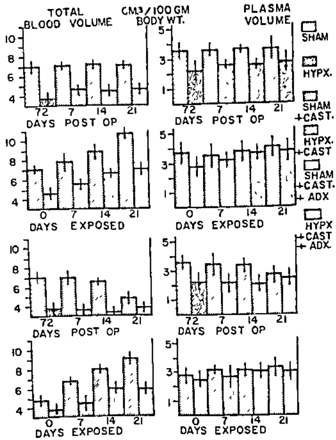


FIGURE 9 Effects of low pressure upon the blood volume changes of castrate-hypophysectomized, castrate-sham-hypophysectomized, castrate-adrenalectomized-hypophysectomized, and castrate adrenalectomized sham-hypophysectomized rats Vertical lines through the bars represent ±1 standard error of the mean

Experiment 4

Peripheral blood FIGURE 6 and TABLES 6 and 11 show peripheral blood studies of castrate-adrenalectomized-hypophysectomized and castrate-adrenalectomized control animals Castration plus adrenalectomy in both groups results in depressions in red cell, hemoglobin, and hematocrit determinations. In addition, reticulocyte percentages of castrate-adrenalectomized controls are significantly elevated (FIGURE 6) Mean corpuscular

volume values reveal reductions ... those observed in pituitary-ablated animals (compare FIGURES 8 and 9) By 21 days postcastration plus adrenalectomy, the hypophysectomized

cytes upon exposure to lowered pressures, significant decreases in blasts and neutrophilic forms are observed in the castrate-adrenalectomized controls when compared to the unexposed castrate-adrenalectomized control rats No significant differences are noted in comparing castrate-adrenalectomized-hypophysectomized and castrate-adrenalectomized control groups exposed to the erythropoietic stimulus employed The imposition of adrenalectomy

TABLE 14
EFFECTS OF LOW PRESSURE UPON THE ADRENAL GLANDS, SPLEEN, AND THYMUS WEIGHTS/100 GM BODY WEIGHT OF PLURIGLANDULAR-DEFICIENT-HYPOPHYSECTOMIZED, AND CONTROL RATS
(Means ± Standard Errors)

	Spleen wt , mg	Thymus wt mg	Adrenal wt mg		Pituitary mg
			Right	Left	
Hypx (72 days postop)					
No exposure (20)*	136 3 ± 16 1	97 9 ± 10 0	3 7 ± 1 0	4 2 ± 1 1	
Exposed 21 days (20)	141 0 ± 15 9	69 4 ± 5 9	4 4 ± 1 3	4 3 ± 1 3	
Hypx (72 days postop) + Adx (21 days postop)					
No exposure (15)	156 3 ± 17 2	117 5 ± 8 7			
Exposed 21 days (15)	124 6 ± 17 8	109 5 ± 11 0			
Hypx (72 days postop) + Cast (21 days postop)					
No exposure (15)	147 0 ± 10 9	105 0 ± 7 9	3 6 ± 1 4	4 0 ± 1 2	
Exposed 21 days (15)	126 3 ± 14 3	86 9 ± 4 8	4 3 ± 0 7	4 0 ± 0 9	
Hypx (72 days postop) + Cast + Adx (21 days postop)					
No exposure (15)	120 2 ± 13 0	100 0 ± 7 4			
Exposed 21 days (15)	108 8 ± 18 9	74 8 ± 9 4			
Controls (72 days postop)					
No exposure (10)	319 0 ± 18 0	101 9 ± 9 9	10 0 ± 1 6	10 4 ± 1 5	3 0 ± 0 3
Exposed 21 days (10)	338 0 ± 20 9	84 6 ± 11 0	15 7 ± 1 1	15 9 ± 1 7	3 2 ± 0 2
Controls (72 days postop) + Adx (21 days postop)					
No exposure (10)	284 7 ± 15 0	100 7 ± 9 5			2 8 ± 0 1
Exposed 21 days (10)	400 4 ± 14 9	189 0 ± 4 9			2 7 ± 0 3
Controls (72 days postop) + Cast (21 days postop)					
No exposure (10)	289 9 ± 12 7	110 2 ± 7 8	12 0 ± 1 2	12 1 ± 1 5	3 2 ± 0 3
Exposed 21 days (10)	292 8 ± 18 7	57 2 ± 10 1	15 0 ± 1 0	15 5 ± 1 3	2 5 ± 0 2
Controls (72 days postop) + Cast + Adx (21 days postop)					
No exposure (10)	299 4 ± 19 9	73 5 ± 5 2			2 6 ± 0 2
Exposed 21 days (10)	393 1 ± 14 4	158 5 ± 4 9			3 1 ± 0 1

* Number of rats

in exposed castrate-hypophysectomized rats does cause a significant rise in lymphocytic marrow values

Organ weights TABLE 14 indicates reduced thymic weights in castrate-adrenalectomized controls as compared to unoperated and castrate-sham-hypophysectomized animals Hypophysis removal results in a decrease in spleen weights and an increase in thymus weights in castrate-adrenalectomized animals when compared to castrate-adrenalectomized-sham-hypophysectomized rats Lowered barometric pressures evoke increases in spleen and thymus weights in castrate-adrenalectomized controls, while splenic and

Bone marrow studies. TABLE 13 indicates that castration plus adrenalectomy in control rats results in a depression of nucleated erythrocytes accompanied by significant elevations in lymphocytes and neutrophils, both young and mature forms The depression in nucleated erythrocyte percentages

TABLE 13
EFFECTS OF LOW PRESSURE ON BONE MARROW CELLULAR PERCENTAGES IN PLEURIGLANDULAR-DEFFICIENT-HYPOPHYSECTOMIZED AND CONTROL RATS
(Mean ± Standard Errors)

	Blasts	Neutrophils*		Eosinophils	Lymphocytes	Nucleated erythrocytes
		Young	Mature			
Hypx (72 days postop)						
No exposure (20)†	0.8 ± 0.1	10.1 ± 1.0	30.5 ± 3.0	5.1 ± 1.0	21.1 ± 1.5	20.0 ± 3
Exposed 21 days (20)	0.9 ± 0.1	6.6 ± 0.8	25.4 ± 1.5	4.4 ± 0.5	10.7 ± 2.0	50.1 ± 2
Hypx (72 days postop) + Adx (21 days postop)						
No exposure (15)	1.2 ± 0.2	7.0 ± 0.9	25.8 ± 1.2	3.5 ± 0.4	42.0 ± 4.2	17.5 ± 2
Exposed 21 days (15)	0.9 ± 0.2	6.2 ± 0.9	26.6 ± 1.1	4.7 ± 0.6	14.6 ± 2.3	45.3 ± 2
Hypx (72 days postop) + Cast (21 days postop)						
No exposure (15)	1.5 ± 0.3	11.0 ± 1.2	30.0 ± 1.7	5.0 ± 0.9	27.0 ± 1.9	24.0 ± 2
Exposed 21 days (15)	1.0 ± 0.2	7.8 ± 0.8	28.9 ± 1.8	4.1 ± 0.5	7.6 ± 2.0	49.2 ± 3
Hypx (72 days postop) + Cast + Adx (21 days postop)						
No exposure (15)	1.2 ± 0.2	6.0 ± 0.7	21.8 ± 2.2	3.5 ± 0.7	44.0 ± 5.0	21.5 ± 2
Exposed 21 days (15)	0.4 ± 0.1	6.8 ± 1.0	25.8 ± 1.9	3.6 ± 1.0	17.0 ± 1.9	44.0 ± 2
Controls (72 days postop)						
No exposure (10)	1.6 ± 0.3	7.4 ± 1.1	33.0 ± 1.0	6.0 ± 1.2	12.6 ± 2.0	35.2 ± 1
Exposed 21 days (10)	0.8 ± 0.3	5.3 ± 0.9	25.1 ± 1.5	3.4 ± 0.8	10.2 ± 2.4	53.1 ± 3
Controls (72 days postop) + Adx (21 days postop)						
No exposure (10)	1.2 ± 0.1	11.6 ± 0.7	36.5 ± 0.8	6.4 ± 1.0	18.2 ± 1.2	23.1 ± 1
Exposed 21 days (10)	0.8 ± 0.2	5.9 ± 0.9	27.0 ± 1.6	5.4 ± 0.5	13.2 ± 1.7	44.8 ± 2
Controls (72 days postop) + Cast (21 days postop)						
No exposure (10)	0.7 ± 0.1	8.0 ± 1.1	34.1 ± 1.4	5.1 ± 1.2	12.8 ± 1.8	33.7 ± 2
Exposed 21 days (10)	0.7 ± 0.4	5.7 ± 0.9	27.1 ± 2.2	3.5 ± 1.2	11.8 ± 2.5	48.7 ± 2
Controls (72 days postop) + Cast + Adx (21 days postop)						
No exposure (10)	1.6 ± 0.2	10.5 ± 0.5	39.4 ± 2.0	3.8 ± 1.0	17.2 ± 1.0	25.0 ± 2
Exposed 21 days (10)	0.6 ± 0.1	5.8 ± 1.2	25.8 ± 1.8	3.0 ± 0.5	16.2 ± 1.7	45.8 ± 3

* Young neutrophils included promyelocytes and myelocytes, mature neutrophils included meta-myelocytes and segmented forms
† Number of rats

seen in castrate-adrenalectomized-hypophysectomized rats is not significantly lower than that observed in similarly operated sham-hypophysectomized controls However, when compared to operated sham-hypophysectomized controls, the myelograms of these animals reveal a marked rise in lymphocytic forms and significant decreases in immature and mature neutrophilic types

The marrow cellular percentages of castrate-adrenalectomized controls subjected to lowered barometric pressures reveal no differences from exposed castrate controls However, apart from the increases in nucleated erythro

tends to raise red cell counts [18]

Apart from the changes in organ weights cited for the hypophysectomized rat, no further changes were noted in any of the pleuriglandular-deficient-hypophysectomized groups Adrenalectomy has been reported to result in an increase in the absolute weight of the lymphoid tissues [15] This seems doubtful from our previous and present work and from the studies of Stoerk,[19] which demonstrated no significant differences in lymphoid weights in adrenalectomized intact animals That adrenalectomy serves to protect the lymphoid tissues against those influences that would produce involution[20] does not appear to be applicable in the adrenalectomized-hypophysectomized rat after exposure to lowered barometric pressures Instead, atrophy of splenic and thymic organ was apparent The well-established findings that hypertrophy of the adrenal gland accompanies the response of animals subjected to low pressures[1] has been confirmed in our experiments

Crafts[21] reported that combined thyroidectomy and adrenalectomy in adult female rats induced an anemia equal in severity to that of hypophysectomy, while Contopoulos *et al* [22] found the posthypophysectomy anemia to be more severe than combined thyroidectomy and adrenalectomy Recent studies by Meineke and Crafts[23] tend to agree with the findings of Contopoulos' group Landau and Gordon[24] have reported that the hypophysectomy effects on blood may be due to the generally reduced oxidative metabo-

costerone has been found to produce a moderate gain in red cell count by the fourth week, but with no significant repair of the posthypophysectomy marrow hypoplasia [25] In the present studies, long-lasting desoxycorticosterone (Percorten) injected in 2 5-mg amounts was found to increase the survival time of operated animals with no apparent effects on the blood picture

It seems clear from the present investigation that the pituitary, adrenal, and ovarian glands are not of primary importance in erythropoiesis, since hypoxia induced by lowered barometric pressures invokes peripheral and bone marrow erythroid responses in pleuriglandular operated animals Our previous findings on the exposed adrenalectomized rats[6] and those of Feigin and Gordon[1] on the exposed hypophysectomized rat have been confirmed and extended

For the most part the anemia of hypophysectomy appears to be normochromic and normocytic This is in accord with the reported findings of Crafts [26] The present results indicate that at the 14-day postoperative period in the adrenalectomized-sham-hypophysectomized and adrenalectomized-hypophysectomized rats, the anemia is of the normochromic macrocytic variety Castration in the hypophysectomized and sham-operated animals results in no differences from the unoperated group However, the

thymic weight decreases occur in the castrate-adrenalectomized-hypophy sectomized group

Discussion

Recently the relationship of the endocrine glands to the formed elemen of the blood and to the blood-forming organs has been reviewed by Gordon The existence of an anemia in rats following pituitary ablation has be established definitely [11-14] The observed decrease in reticulocytes in t hypophysectomized rat seems to indicate a decreased rate of erythrocy production and delivery to the blood stream Our studies reveal that, addition to the observed lowered concentration of erythrocytic elements, t marrow of the hypophysectomized rat is characterized by an increase in t percentages of young neutrophilic and lymphocytic forms

Evidence has been accumulating steadily to show that endocrines ex an influence upon the white blood cell elements [10, 15] Our findings of increase in peripheral white cell counts in adult female rats with a tenden toward neutrophilia and lymphopenia are in accord with those of Craft in male rats Eosinophil counts were found to be increased significant In view of the fact that hypoadrenocorticalism plays a role in posthypop ysectomy changes and that adrenal insufficiency is often associated w ... y ly ... aden ... es, ... been observed Dougherty,[16] in a survey of the literature, revealed th ...

... adrenalectomized, and castrate-ad ... In this respect, further decreases ... arameters below plateau hypophys tomy levels with elevated reticulocyte percentages were noted in both ad nalectomized pleuriglandular deficiency groups In the adrenalectomiz ...

In addition to the depressed nucleated erythrocytes, adrenalectomy evok

Acknowledgments

I am indebted to R. D Collins, C E. Pilliero, and E Smith for their technical assistance during the course of this investigation Acknowledgment is made of the suggestion by A S Gordon for the use of Percorten in some phases of this investigation

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imposition of adrenalectomy in castrate-hypophysectomized animals produces a microcytosis Macrocytic hypochromic erythrocytes are characteristic of all the exposed adrenalectomized groups, while the remaining endocrine-deficient exposed animals reveal normochromic normocytic cells

The anemias characteristic of hypophysectomy, adrenalectomy, and pleuriglandular deficiency are the result of decreases in erythroid elements with no concomitant plasma volume changes This is apparent from the data and calculations of total red cell counts per total blood volume Elevations in erythrocyte numbers are substantiated by our finding that blood volume levels are significantly increased after exposure to the hypox stimulus employed

The hypertrophy of the adrenal glands occurring under low-pressure stimulation in sham-operated animals has been reported previously[1, 4] and confirmed by our present experiments Cortical hypertrophy fails to occ in the exposed hypophysectomized intact groups, the cortices being in a atrophic state This supports the large mass of evidence which demo strates that cortical hypertrophy may be under pituitary regulation.

That hypoxia is the fundamental stimulus for erythropoiesis has be ... accepted by most investigators[27] However, the fundamen ... chronoic ers org

is probable that the endocrine ... are secondary manifestations of their influence upon metabolic reacti occurring elsewhere In this regard, Gordon[10] has suggested that the en ... may act as a buffer mechanism, serving to adjust the quality ... rements

· Co ituit is on not

circulating plasma erythropoietic factor(s), ... support to this view

Summary and Conclusions

An intensive study and analysis of the effects of pleuriglandular defici on the peripheral blood, blood volume levels, and bone marrow cyt has been made

That the pituitary, adrenal, and ovarian glands are not of primary in tance in erythropoiesis is indicated by the results of the present investiga

It seems clear that the endocrine system plays a role in controllin ... of ... within the circulation.

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ACKNOWLEDGMENTS

I am indebted to R. D. Collins, C. E. Piliero, and E. Smith for their technical assistance during the course of this investigation. Acknowledgment is made of the suggestion by A. S. Gordon for the use of Percorten in some phases of this investigation.

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imposition of adrenalectomy in castrate-hypophysectomized animals pro

The anemias characteristic of hypophysectomy, adrenalectomy, a pleuriglandular deficiency are the result of decreases in erythroid eleme with no concomitant plasma volume changes. This is apparent from t data and calculations of total red cell counts per total blood volume Ele tions in erythrocyte numbers are substantiated by our finding that bl volume levels are significantly increased after exposure to the hypo stimulus employed

The hypertrophy of the adrenal glands occurring under low-pressure stin lation in sham-operated animals has been reported previously[1,4] and confirmed by our present experiments Cortical hypertrophy fails to oc in the exposed hypophysectomized intact groups, the cortices being in atrophic state This supports the large mass of evidence which dem strates that cortical hypertrophy may be under pituitary regulation

That hypoxia is the fundamental stimulus for erythropoiesis has b generally accepted by most investigators.[27] However, the fundamen question as to how the stimulus of hypoxia results in increased erythropoi so far has evaded solution In the light of our studies and those of others is probable that the endocrine effects exerted upon hematopoietic org are secondary manifestations of their influence upon metabolic reacti occurring elsewhere In this regard, Gordon[10] has suggested that the en crines may act as a buffer mechanism, serving to adjust the quality a intensity of the erythropoietic reaction in response to the requirements oxygen by the body cells The revived view has been put forth by Cor poulos and his group[28-31] that a specific hormone produced by the pituit is essential for erythropoiesis Our present studies and investigations on circulating plasma erythropoietic factor(s), erythropoietin(s), do not l support to this view.

Summary and Conclusions

An intensive study and analysis of the effects of pleuriglandular deficie on the peripheral blood, blood volume levels, and bone marrow cytol has been made

Histological studies of the morphological responses of the pituita adrenal, spleen, and thymus glands are now in progress.

It is probable that the endocrine effects exerted upon hematopoietic org are secondary manifestations of their influence upon metabolic reacti occurring elsewhere.

THE PITUITARY ERYTHROPOIETIC FACTOR

Donald C. Van Dyke

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Although the anterior pituitary is known to influence erythropoiesis through several of its trophic hormones,[1,2] I shall not attempt to review these effects. Instead, I shall restrict this discussion to two specific questions. Is there adequate evidence for proposing the existence of a discrete pituitary erythropoietin? Is there reason to consider the pituitary as the tissue of origin of the erythropoietin that is concentrated from plasma and urine of anemic animals?

In spite of considerable efforts made to separate a discrete erythropoietin from the anterior pituitary, no such separation has been accomplished[3] and,

TABLE 1

ADRENAL WEIGHT STIMULATING, ERYTHROPOIETIC, AND CALORIGENIC DOSE RESPONSE RELATIONSHIP OF α CORTICOTROPIN

(Six Hypophysectomized Rats per Group, Injection in Beeswax)

Dose (µg/day 14 days)	Hemoglobin (gm/100 ml)	Hematocrit (percentages)	RCV/100 gm (ml)	Adrenals (mg)	Thymus (mg)	Metabolic rate (Cal/sq m/hr)
50	11.7	43.5	2.11 ± .03*	35.0	Atrophic	36.9 ± 1.9*
25	13.7	48.4	2.18 ± .01	25.3	Atrophic	31.1 ± 1.5
10	11.6	37.2	1.88 ± .04	11.5	Atrophic	24.4 ± 1.9
5	9.6	30.6	1.52 ± .04	8.5	82	20.1 ± 0.6
H Control	9.0	27.7	1.43 ± .02	6.0	81	19.0 ± 0.9

* Standard error of the mean.

since highly purified α-corticotropin is the most potent erythropoietin that has been obtained from the pituitary, for all practical purposes, the terms corticotropin and "pituitary erythropoietic factor," must be considered synonymous. TABLE 1 illustrates the erythropoietic activity of a pure α-corticotropin prepared by C. H. Li *et al.*[4] As little as 10 µg/day for 14 days produced a significant increase in hemoglobin, hematocrit, and circulating red cell volume.

Although the corticotropic activity of crude pituitary fractions can be diminished greatly by autolytic digestion without seriously affecting the erythropoietic activity, preliminary evidence suggests that controlled digestion of a highly purified α-corticotropin may give similar results. A com-

that resulting from combined thyroidectomy, adrenalectomy and gonadectomy Endocrinology 55. 509
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anterior lobe of sheep pituitary[7] and, later, by injecting "purified pituitary preparations containing ACTH but relatively free of other pituitary hormones"[8] into hypophysectomized rats These experiments seemed to establish the fact that extracts containing corticotropin will stimulate erythropoiesis in the absence of the adrenal, and attempts to separate an erythropoietically active principle from corticotropin were undertaken, using hypophysectomized animals for the assay

A recent re-examination of the effectiveness of pituitary extracts in the absence of the adrenal was made using the Fe^{59}-red cell incorporation assay of Fried *et al*[10] A pituitary extract prepared by the method of Astwood

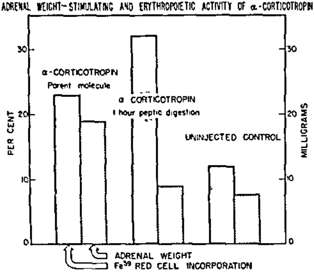


FIGURE 2 A comparison of the adrenal weight stimulating and erythropoietic activity of α-corticotropin before and after peptic digestion

et al[11] for the concentration of corticotropic activity, which was effective in increasing Fe^{59} incorporation into red cells in hypophysectomized rats, failed to produce an increase in hypophysectomized-adrenalectomized rats The rats were given 2 mg/day for 3 days Twenty-four hours after the third injection, Fe^{59} was given intravenously, and the blood was taken for counting 16 hours later The results are summarized in TABLE 2 Unless an erythropoietic effect of corticotropin in the absence of the adrenals can be demonstrated repeatedly, the concept of a discrete pituitary erythropoietin must be abandoned

Recently Fisher[12] has demonstrated that administration of hydrocortisone or corticosterone for 60 days to normal rats elevated the total circulating red cell volume to 1 5 times that of normal controls, thus establishing the importance of corticotropin, mediated through adrenal cortical steroids, in erythropoiesis.

produced equal increases in circulating red cell volume, but varied greatly in adrenal weight-stimulating activity. FIGURE 2 compares the adrenal weight-stimulating activity and Fe^{59} incorporation of highly purified α-corticotropin with that of α-corticotropin subjected to peptic digestion for 1 hour, as assayed in hypophysectomized rats 7 days p o (this work was done in collaboration with C H Li, University of California, Berkeley, Calif.) The decrease in the adrenal weight-stimulating activity of corticotropin with retention of its erythropoietic activity was previously interpreted as evidence for contamination of corticotropin with erythropoietin.[4] Thus the concept of a discrete pituitary erythropoietin depends largely on biological evidence

Previously presented arguments against the identity of corticotropin and pituitary erythropoietin made on a biological basis[4] have included the fact

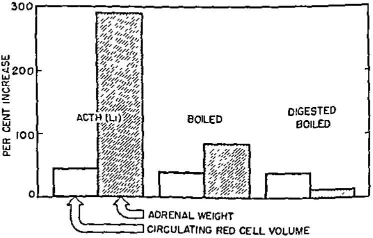


FIGURE 1 A comparison of the adrenal weight-stimulating and erythropoietic activities of highly purified corticotropin, undigested boiled extracts, and autolytically digested pituitary extracts

that corticotropin has little effect on the noninjected partner in a parabiotic cross-transfusion experiment whereas, when one member is subjected to hypoxia, the erythropoietic effect is easily demonstrated in the partner.[4] Such arguments must yield to the realization that another, nonpituitary mechanism is unquestionably involved Thus the biological evidence is reduced to two observations: (1) that corticotropin will stimulate erythropoiesis in the absence of the adrenals,[7, 8] and (2) that the adrenalectomized [illegible]

[illegible]

depends on the adequacy of the demonstration that administered corticotropin will stimulate erythropoiesis in the adrenalectomized animal. Such evidence was first obtained by feeding adrenalectomized animals a diet of

tions,[14] thyrotropic hormone,[14] and α-corticotropin,[6, 14] as well as l-thyroxin in normal rats. As can be seen from the figure, the correlation is unquestionable. It has been shown that corticotropin has no calorigenic effect in the absence of the adrenals,[14] but no data are available comparing the erythropoietic and the metabolic effects of pituitary extracts in hypophysectomized-adrenalectomized rats.

The calorigenic effect of adrenal steroids in hypophysectomized rats[14] suggests an explanation for the altered ratio of adrenal weight-stimulating and erythropoietic activity of digested corticotropin preparations. The decrease in adrenal weight-stimulating activity of corticotropin with retention of

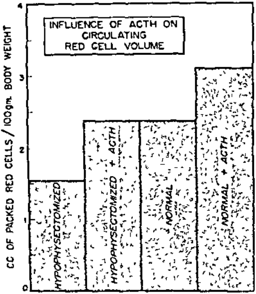


FIGURE 4. A demonstration of the erythropoietic activity of corticotropin after long term (116 days) administration to either hypophysectomized or normal rats.

erythropoietic activity following digestion was interpreted previously as possible evidence for the existence of two separate substances. However, a decrease in adrenal weight-stimulating activity does not ensure a decrease in ability to stimulate cortical steroid output.

Both in its biological and chemical characteristics, corticotropin differs from the plasma and urinary erythropoietin. Corticotropin is a low molecular weight polypeptide,[6] whereas the erythropoietins of plasma and urine appear to be nondialyzable mucoproteins.[15, 16] Rambach and his co-workers have recently found that the active fraction from plasma contains a large amount of carbohydrate with a small amount of nitrogen,[17] and we have found that the urinary erythropoietin is in the nonultrafiltrable fraction of urine, which contains about 50 per cent carbohydrate.[18] Furthermore, the

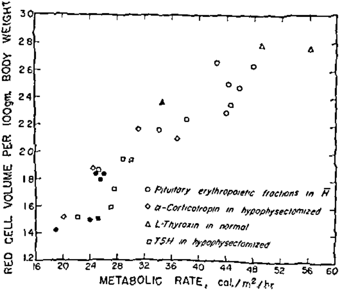
When the red cell volume is plotted against the metabolic rate of hypophy-sectomized rats injected with corticotropin or corticotropin-containing pitui-tary extracts, the correlation indicates clearly that the calorigenic activity of corticotropin, mediated through the steroids of the adrenal cortex, i

Table 2

Failure of "Pituitary Erythropoietic Hormone" to Increase Fe^{59} Incorporation in the Absence of the Adrenals

Treatment	Recipient	Fe^{59} Uptake (percentage)
"EH"*	Hypophysectomized adrenalectomized	6.5 ± 1.8†
Beeswax	Hypophysectomized adrenalectomized	6.6 ± 1.4
"EH"	Hypophysectomized	16.8 ± 2.5‡
Beeswax	Hypophysectomized	4.5 ± 1.5

* Administered in beeswax
† Standard error of the mean
‡ $p = 0.01$



volume and metaboli
treatment. The soli

unquestionably an adequate explanation for the erythropoietic activity o corticotropin in the hypophysectomized rat FIGURE 3 compares th available published data on standard metabolic rate and red cell volum for hypophysectomized rats injected with "pituitary erythropoietic frac

increasing the adrenal weight, producing atrophy of the thymus, or increasing the metabolic rate [11]

These striking differences in both the chemical and biological characteristics of corticotropin and plasma or urinary erythropoietin, plus the demonstrations by Fried et al [19] and by Crafts and Meineke[1] that the level of erythropoietin is elevated in the plasma of bled hypophysectomized rats, would seem to rule out the pituitary as an important contributor of any erythropoietin found in the body fluids

Summary

Both in its biological and chemical characteristics, corticotropin differs from the erythropoietin found in plasma or urine These differences plus the demonstration that the level of erythropoietin is elevated in the plasma of bled hypophysectomized rats rule out the pituitary as an important contributor to any erythropoietin found in the body fluids

The erythropoietic activity of corticotropin or corticotropin-containing pituitary extracts in normal and hypophysectomized rats can be explained as secondary to the erythropoietic activity of the adrenal steroids

The concept of a discrete pituitary erythropoietic hormone depends on the adequacy of the demonstration that corticotropin administration will stimulate erythropoiesis in the absence of the adrenals Although evidence has been previously presented indicating that the adrenals are not essential for the erythropoietic response to corticotropin, recent evidence suggests that the adrenals may be essential Unless an erythropoietic effect of corticotropin can be repeatedly demonstrated in the absence of the adrenals, the concept of a discrete pituitary erythropoietic hormone must be abandoned

In spite of considerable efforts to separate a discrete erythropoietin from the anterior pituitary, no such separation has been accomplished, and since corticotropin is the most potent erythropoietin that has been obtained from the pituitary, the terms corticotropin and "pituitary erythropoietic hormone" must be considered synonymous

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urinary erythropoietin loses 90 per cent of its activity upon being oxidi with periodate, which is consistent with its suggested mucoprotein naturi

Erythropoietically active pituitary extracts that will increase readily red cell volume of hypophysectomized rats have been far less effective w administered to normal rats The original observation by Garcia et that corticotropin would produce a polycythemia in normal rats[17] was m after 116 days of injection (FIGURE 4), after only 30 days of injection,

TABLE 3*
ERYTHROPOIETIC ACTIVITY OF PLASMA OF PHENYLHYDRAZINE-TREATED AND NORMAL RABBITS ASSAYED IN HYPOPHYSECTOMIZED RATS*

Treatment	Hemoglobin (gm 100 ml)	Hematocrit (percentages)	RCV/100 gm (ml)	Adrenal wt (mg)	Thymus (mg)
Anemic	13 5	46 3	2 13 ± 0 12†	7 3	101
Normal	9 2	29 4	1 38 ± 0 09	6 3	92
Uninjected	10 0	31 1	1 41 ± 0 09	7 3	119

* Two ml day for 14 days
† Standard error of the mean

TABLE 4
HEMATOLOGICAL VALUES, ORGAN AND BODY WEIGHTS, AND METABOLIC RATE NORMAL RATS INJECTED WITH URINARY ERYTHROPOIETIN OR EXPOSED TO SIMULATED ALTITUDE OF 15,000 FEET FOR 14 DAYS

Treatment	Total red cell volume (ml)	Hematocrit (percentages)	Body wt (gm)	Adrenal wt (mg)	Thymus wt (mg)	Metabolic (Cal /sq m
Urine concentrate	6 70 ± 83*	56 8 ± 4 24*	171	59	211	34 4
Hypoxia	5 76 ± 46	49 9 ± 1 95	171	51	192	—
Saline	4 48 ± 55	43 0 ± 1 66	171	50	245	31 5

* Standard error of the mean

response was obtained On the other hand, the plasma and urinary ery

filtrate of active plasma from phenylhydrazine-treated rabbits was obta (from Keighley).[20]

per cent increase in the total circulating red cell volume, again wit

STUDIES OF ERYTHROPOIETIN. THE HORMONE REGULATING RED CELL PRODUCTION

Leon O Jacobson, Eugene Goldwasser, Clifford W Gurney, Walter Fried, Louis Plzak

The Argonne Cancer Research Hospital, and the Departments of Medicine and Biochemistry University of Chicago, Chicago, Ill*

There is mounting evidence that erythropoiesis is controlled by a humoral factor or factors Since Carnot and Déflandre[1] first published this thesis in 1906, many investigators have attempted to substantiate their work, but it has been largely in the last twenty years that such investigators as Reissmann,[2] Erslev,[3] Gordon and his co-workers,[4] Borsook *et al*,[5] and Stohlman *et al*[6] have succeeded in bringing the problem to the point of serious study and acceptance In 1952 Grant and Root[7] wrote a comprehensive review of the literature on the subject

The first convincing support of Carnot's hypothesis is found in the work of Reissmann,[2] and Stohlman *et al*[6] Reissmann demonstrated that increased erythropoiesis occurred in both parabionts even though only one of the pair was maintained in low O_2 tension Stohlman and Rath found that erythroblastic hyperplasia was not confined to the areas of regional hypoxia in a human being with regional hypoxia secondary to a patent ductus arteriosus Hyperplasia was also observed in the bone marrow that was supplied by blood with a normal O_2 saturation

Of necessity, the relationship of the humoral factor that is present in anemic plasma to the maintenance of a normal amount of red blood cells and hemoglobin has been one of conjecture until recently, when Gurney and his associates demonstrated its presence in normal plasma[8] The exact relationship of arterial oxygen saturation to the production of circulatory erythropoietin† and, thus, to erythropoiesis has likewise not been established

During the past several years we have been concerned with (1) applying the technique of Fe^{59} incorporation as a measure of red cell production, (2) finding more sensitive assay preparations for erythropoietin, (3) elucidating the role of erythropoietin in maintaining the dynamic equilibrium of erythropoiesis (4) determining the basic conditions that control erythropoietin blood levels and, thus, the rate of erythropoiesis, (5) studying the effect of cobaltous ion on erythropoietin production, (6) searching for the site of erythropoietin production, and, of course, (7) using the information obtained to solve certain clinical problems A brief discussion of our research and some interpretations of the data are the subjects of this paper

* Operated by the University of Chicago for the United States Atomic Energy Commission

† Erythropoietin is used in this text to mean any factor found in plasma that, when given to test animals will stimulate (1) incorporation of Fe^{59} into red cells or (2) reticulocyte formation or (3) will increase the size of the red cell mass The possibility that there may be more than one factor is not excluded

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Studies of Hypophysectomized Rats

Because we were investigating recovery from radiation injury and leaned toward the concept that the mechanism of recovery of irradiated animals injected with cells from a hematopoietic source was humoral in nature,[13] we became interested in the work of Van Dyke *et al.*[14] These investigators found that, after rats were hypophysectomized, the red cell mass fell to about one half its original value within the next three months. In addition, they demonstrated that the red cell mass could be restored in the hypophysectomized animals by giving them a pituitary extract. On the basis of these and other findings, they postulated that the hypophysis is involved in the elaboration of a factor that has a direct effect upon erythropoiesis.

Effect of Hypophysectomy on Incorporation of Fe^{59}

We found that by 4 days after hypophysectomy red cell production, as measured by Fe^{59} incorporation, was reduced and reached a minimum at 8 or more days after the operation (FIGURE 1). It was of interest that red cell production fell more slowly and stabilized at a higher minimum value in young rats (about 4 weeks of age and weighing 75 to 90 gm.) than in older ones (FIGURE 1). In both age groups a minimum uptake was reached between 8 and 21 days, thereafter the uptake of iron rose slowly to about 50 per cent of that of the original value.

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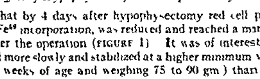


FIGURE 1. Percentage incorporation of Fe^{59} into red blood cells of rats hypophysectomized at 4 or 8 weeks of age.

Fe^{59} Red Cell Incorporation as a Measure of Erythropoietin Activity of Plasma

The work of Huff and his group[9] demonstrated the usefulness of the measurement of Fe^{59} incorporation into newly formed red cells as a means of determining the rate of erythropoiesis We used their technique, with a few modifications, to study the effects of anemic and normal plasma on erythropoiesis The method we use[10, 11] involves the intravenous injection of the test material into rats, followed by the injection of Fe^{59}. When there is no longer the possibility that Fe^{59} will be found in the plasma, a sample of blood is withdrawn and counted in a well-type scintillation counter. From the

TABLE 1
EFFECT OF ANEMIC PLASMA ON Fe^{59} INCORPORATION INTO THE RED BLOOD CELLS OF NORMAL RATS*

	Number of rats	Percentage incorporation
Saline	4	36 5
	3	37 5
	4	33 5
	3	32 5
	5	32 5
Anemic plasma	4	46 0
	5	45 5

* Each animal was given 3 injections of 2 ml each of either saline or anemic plasma at daily intervals

the animal
corporation
used in each
test group, and the results are expressed as the average percentage incorporation. An example of the range of incorporation of Fe^{59} after administration of anemic plasma and saline to normal rats is given in TABLE 1 The values for rats injected with normal plasma are comparable to those for rats injected with saline.

The Fe^{59} procedure was found to be much simpler, more reliable, more sensitive, and less time-consuming than the methods used previously for

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of
these methods, including Fe^{59} red cell incorporation, can a significant difference be found between control and anemic plasma, if the anemic plasma being tested has only a relatively small increased erythropoietin titer above normal For this reason, efforts were made to find more sensitive methods for assay

Studies of Hypophysectomized Rats

Because we were investigating recovery from radiation injury and leaned [illegible] mechanism of recovery of irradiated animals [illegible]

Effect of Hypophysectomy on Incorporation [illegible]

[illegible] after hypophysectomy red cell production, a[illegible]

Effect of Hypophysectomy on the Number of [illegible]

[illegible] the number of reticulocytes in the peripheral blood of hypophy[illegible] were comparable to [illegible] red cells Within [illegible] culocytes fell by a

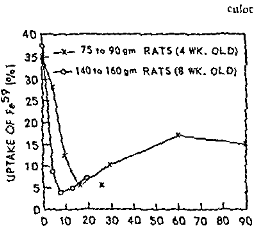


FIGURE 1 Percentage incorporation of Fe^{59} into red blood cells of rats hypophysectomized at 4 or 8 weeks of age

factor of at least 10, and then rose slowly. The effect of age at the time o hypophysectomy on the rapidity and extent of reticulocyte depression wa also quite apparent (FIGURE 2).

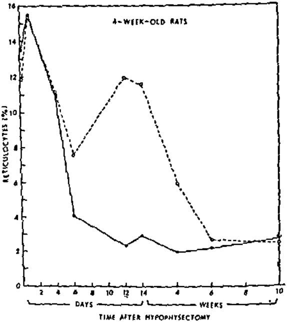


FIGURE 2 Effect of hypophysectomy on the reticulocyte values of 4-week-old ra Hypophysectomized, ————, control, - - - -

Effect of Anemic Plasma on the Incorporation of Fe^{59} into Red Cells of Hypophysectomized Rats

If the hypophysectomized young adult rat is used to assay anemic plasi at a time when its erythropoietic rate is at a minimum, the magnitude of response is considerably greater than that of the normal animal (TABLE In the normal rat the average ratio of the stimulated rat to the cont

Production of Erythropoietin by Hypophysectomized Animals

Finkelstein *et al* [15] have shown that hypophysectomized rats develo reticulocytosis in response to bleeding. We have found that plasma fr hypophysectomized rats in which the hematocrit has been reduced to 25 cent or less by repeated bleeding produces a significant increase in the cell incorporation of Fe^{59} in hypophysectomized assay preparations (TABLE

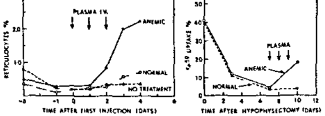


FIGURE 3 Effect of anemic plasma on the incorporation of Fe^{59} and on the reticulocyte values of hypophysectomized rats

TABLE 2
EFFECT OF ANEMIC PLASMA ON RATE OF ERYTHROPOIESIS IN THE HYPOPHYSECTOMIZED RAT SIX EXPERIMENTS*

No rats per group	Average percentage uptake of Fe^{59} into RBC			
	Anemic plasma	Normal plasma	Saline	No injection
5	27 4	3 2	5 0	4 0
5	16 3	3 3		
4	17 1	5 0		
5	18	2 7	3 8	
3	19 3	5 0		
3	21 8	5 0		

* 10 to 15 days posthypophysectomy

TABLE 3
EFFECT OF HYPOPHYSECTOMY ON CAPACITY OF ANIMAL TO ELABORATE THE ANEMIC PLASMA FACTOR
Average Percentage uptake of Fe^{59} in RBC of Hypophysectomized Rats after Administration of Various Plasmas

No of rats	Total vol of plasma 2 cc, in)	Source of plasma			
		Unoperated, unbled animal	Hypophysectomized unbled animal	Unoperated anemic animal	Hypophysectomized anemic animal
5	4	2 7	5 9	14 8	12 0
5	4	5 0	5 8	16 7	16 3
5	2			11 2	14 2
13	2			12 4	12 3

It may thus be concluded that (1) the hypophysectomized animal retains the capacity to produce erythropoietin in response to repeated hemorrhage, and that (2) the "exaggerated" response of the hypophysectomized animal probably is due to a decrease in the amount of circulating erythropoietin in that animal Supported by these conclusions, we postulate the following thesis

Within a few days after hypophysectomy, an over-all metabolic reduction occurs As a result of this reduced metabolic requirement, and during the interval before the red cell mass has fallen appreciably, the hypophysectomized animal is more or less comparable to an animal that has been made polycythemic by red cell transfusion Hence the production of erythropoietin, which we suggest is responsible for maintaining the steady state of circulating erythrocytes, is reduced to a minimum because a plethora of red cells exists If this explanation is valid, then transfusion-induced polycythemia or any other condition that produces a relative plethora of red cells should reduce erythropoiesis It has been shown already that transfusion-induced polycythemia reduces erythropoiesis.[16] The metabolism of animals is known to diminish as starvation progresses,[17] and it has been demonstrated that hyperoxia reduces erythropoiesis [18] Therefore, we experimentally induced these conditions in order to learn whether starved, hyperoxic, and transfusion induced polycythemic animals respond to anemic plasma as the hypophysectomized animals do

Experiments with Transfusion-Induced Polycythemic Animals

Erythropoiesis is suppressed in transfusion-induced polycythemic rats or mice to the degree that Fe^{59} incorporation is greatly reduced and the peripheral blood is devoid of reticulocytes The polycythemic animals respon

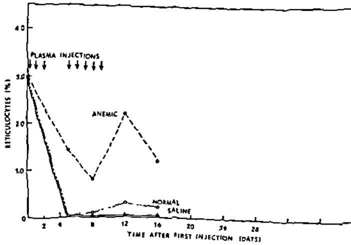


FIGURE 4 Effect of repeated 0 5-cc. intravenous injections of anemic and normal rabbit plasma on the reticulocyte values of mice that received intraperitoneal injections of plasm beginning on the same day that transfusions for polycythemia were initiated

to anemic plasma to a greater degree than normal rats and mice, as is the case with hypophysectomized rats (FIGURE 4)

Experiments with Starved Rats

Four days after a starvation program has been initiated, erythropoiesis is reduced to a minimum in the rat The response of the starved animals to anemic plasma is similar to that of those hypophysectomized (FIGURE 5)

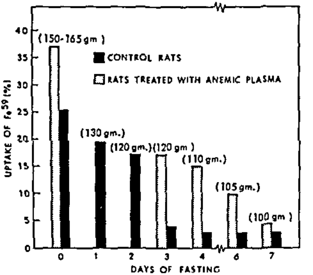


FIGURE 5 Effect of duration of starvation on Fe^{59} incorporation and on response to erythropoietin in 8-week old rats

Experiments with Hyperoxic Rats

Hyperoxia reduces erythropoiesis in rats, and animals in this condition give an exaggerated response to the injection of anemic plasma (TABLE 4)

Factors Controlling Rate of Erythropoiesis and Production of Erythropoietin

The data that have accumulated from studies of hypophysectomized, polycythemic, starved, and hyperoxic rats and mice suggested to us that the dynamic equilibrium of the erythron is regulated by erythropoietin that is present in the circulating blood, and that the quantity of this hormone (and thus the over-all production of erythrocytes) is in some way determined by the relationship of tissue oxygen supply to tissue oxygen demand, rather than by either factor alone [19]

This hypothesis is strengthened by the fact that, in response to anemic anoxia produced by bleeding or by hemolysis induced by the administration of phenylhydrazine, plasma erythropoietin levels are increased. In these circumstances, the available oxygen supply is reduced without any

appreciable alteration in the demand. Dinitrophenol, a metabolic stimulant was tested in normal rats and was found to cause an increase in the rate of incorporation of Fe^{59} into red cells and a decrease in the sensitivity of rats to exogenous erythropoietin (TABLE 4) Another metabolic stimulant, triiodothyronine, was observed to have similar effects. The data that reveal the relationship of oxygen demand and supply and how it affects erythropoiesis[4] are given in TABLE 5.

An understanding of the oxygen supply and demand relationship does not clarify the problem completely An end organ must be able to respond to the changes that are occurring by reducing or increasing the production of erythropoietin to satisfy the *metabolic O_2 requirement* in an equilibrium state The metabolic O_2 requirement might be imagined to be fairly stable in a

TABLE 4
RELATIVE SENSITIVITY OF ASSAY PREPARATIONS TO ANEMIC PLASMA IN TERMS OF PERCENTAGE OF FE^{59} INCORPORATION

	Fe^{59} red cell incorporation in percentage*	
	Anemic	Control
Recipient		
Polycythemic	24	4
Normal	44	32
Hyperoxic	24	8
Normal	46 5	34
Starved (4 days prior to administration of Fe^{59})	15	3
Normal	38	27
Dinitrophenol to normal rat	42	36
Normal	43	22

* Each figure is an average of 5 individual values

[illegible]

response to temporary breakdowns in respiratory and circulatory mechanisms which, though brief, might yet be sufficient to trigger the increased production of erythropoietin With a rapid compensatory response, however, production might be expected to fall quickly, and thus the over-all balance of the [illegible]

TABLE 5
RELATIONSHIP OF OXYGEN SUPPLY AND DEMAND TO ERYTHROPOIESIS

Condition	O_2 supply	O_2 demand	Rate of erythropoiesis	Sensitivity to erythropoietin
Hypophysectomy	Normal	Decreased	Reduced	Increased
Hyperoxia	Increased	Normal	Reduced	Increased
Starvation	Normal	Decreased	Reduced	Increased
Polycythemia*	Increased	Normal	Reduced	Increased
Anemia due to				
Phlebotomy	Reduced	Normal	Increased	Decreased
Phenylhydrazine	Reduced	Normal	Increased	Decreased
Dinitrophenol	Normal	Increased	Increased	Decreased
Triiodothyronine	Normal	Increased	Increased	Decreased

* Induced by red cell injection

to changes in partial pressure of oxygen. If oxygen is involved directly, then it must be the partial pressure of oxygen since, as shown in TABLE 5, arterial oxygen saturation may be normal, yet erythropoietin production or erythropoiesis may rise or fall. The stimulus may not involve oxygen directly. It may be that a metabolic product monitors the oxygen demand-supply relationship for the body as a whole.

Effect of Cobaltous Ion on Production of Erythropoietin

It is well established that Co^{++} increases the rate of erythropoiesis in man and experimental animals, and eventually produces a polycythemia. Early work suggested that cobalt exerted its erythropoietic action by directly producing a condition of anoxia in the marrow.[21] However, this has been proved to be untenable.[22] We investigated the possibility that Co^{++} might exert its erythropoietic effect by increasing the level of erythropoietin in the plasma.

Subcutaneous injections of cobaltous chloride (250 μM/kg.) containing a tracer amount of Co^{60} were given to rats that were exsanguinated 10 hours later. Their plasma was assayed in starved rats. The cobalt content of this "cobalt plasma," based upon the amount of Co^{60} present, was 0.21 μM/ml. Normal plasma with cobaltous chloride added in the same amount was used as the control because cobaltous ion per se increases the rate of Fe^{59} incorporation.[23] The results of this assay (TABLE 6) indicate clearly that the pronounced increase in the incorporation of Fe^{59} that was observed cannot be due to the presence of a small amount of cobaltous ion, but perhaps to the presence of an increased amount of erythropoietin in the plasma. It is possible, however, that a form of cobalt other than cobaltous ion is responsible for the observed effect.

We have some evidence that certain of the properties of erythropoietin in anemic plasma are also common to those of the active factor in cobalt plasma. The erythropoietic activity of both anemic and cobalt plasma is

retained in the soluble fraction after either denaturation of the proteins at 100° C at pH 5 5,[5] or precipitation of the bulk of the plasma protein with 5 per cent perchloric acid [24] It has been reported that erythropoietin is not heat-stable We have found that there is an appreciable loss of activity, but not a complete one The heat-stable, acid-soluble activity of both types of plasma may be dialyzed without marked loss of erythropoietin titer While these parallelisms cannot be accepted as conclusive evidence that the material in cobalt plasma is identical with that in anemic plasma, they suggest that both types of plasma contain erythropoietic factors with grossly similar properties It is premature at this time to hold that there is only one erythropoietin that accounts for the activity produced by the various stimuli When erythropoietin has been characterized more completely, it will be possible to determine whether cobalt plasma contains the factor that is identical with that found in anemic plasma.

TABLE 6
EFFECT OF COBALT PLASMA ON Fe^{59} INCORPORATION INTO THE RED BLOOD CELLS OF STARVED RATS

	Percentage incorporation
Cobalt plasma	13 9 ± 2 6†
Normal plasma 0 21 μM Co/ml	3 1 ± 0 6
Anemic plasma*	15 1 ± 3 3
Normal plasma	2 8 ± 0 9

* From phenylhydrazine-treated rats
† Standard deviation of the mean

The time course of the appearance of erythropoietin in the plasma of rat after injection of cobaltous ion is shown in FIGURE 6 It is evident that a 4 hours after a single injection of Co^{++} the erythropoietin titer started t rise sharply, reaching a maximum at about 12 hours and thereafter declinin sharply

In addition to studying the effect of cobalt as a function of time, we als studied the effect of a single massive bleeding on the erythropoietin titer The data are plotted in FIGURE 6. In this particular experiment, the tite [illegible] stimul

per

of th

The mechanism by which Co^{++} produces erythropoietin and thus increase erythropoiesis is still obscure. While it seems logical that cobalt migh exert its effect by producing an anoxia directly in the sensitive organ tha [illegible]

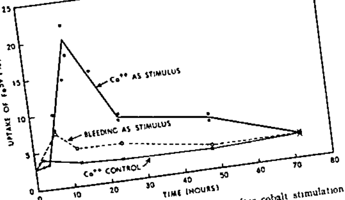


FIGURE 6 Time course of erythropoietin formation after cobalt stimulation Each point represents the average of values obtained from a group of 5 assay rats

The Effect of Protein Depletion on Erythropoiesis and on the Sensitivity to Erythropoietin(s)

A number of investigators have shown that protein depletion causes a reduction in the rate of erythropoiesis Bethard and Wissler *et al* [26] demonstrated that essential amino acid depletion in rats drastically reduced erythropoiesis, as judged by the incorporation of Fe^{59} into red cells Other investigators have shown that the anemia of protein depletion responds favorably to cobaltous ion,[27] and still others have shown that protein-depleted anemic rats retain the capacity to respond to hemorrhage by a reticulocytosis and erythroblastic marrow hyperplasia [28] It thus appears to us that erythropoietin may be the common denominator that causes the reduction in erythropoiesis and the response to such stimuli as cobalt and bleeding in the protein-depleted animal

To test this hypothesis, two groups of rats were placed on special diets The experimental group was given a diet devoid of essential amino acids and the control group was given the same diet with added casein The animals in the amino acid-deficient group experienced a reduction in number of reticulocytes The reduction differed from that of the control group by a factor of at least three by the fifteenth day after the start of the experiment

The rats (in groups of 5) were then given on two consecutive days intravenous injections of 2 ml of (1) anemic plasma, (2) normal plasma, (3) cobalt plasma, (4) anemic plasma extract* (with known erythropoietic activity), (6) cobaltous (5) plasma extract* (known to have no erythropoietic activity), (6) cobaltous chloride, or (7) saline On the day following the second injection, 1 μc of Fe^{59} (as ferric citrate) was injected into the tail vein, and 18 hours later a 1-ml. sample of blood was taken by cardiac puncture and counted in a well-type crystal scintillation counter The percentage of incorporation of the isotope

* Contains 3 mg protein per ml

into red cells was calculated from knowledge of the total body weight, the total amount of radioactivity injected, and the radioactivity per ml of blood. Hematocrits and red blood cell hemoglobin, and reticulocyte determinations were made on the same animals before and after the experiment.

The data reveal that erythropoiesis was depressed markedly in the amino acid-depleted rats, as judged by reduced Fe^{59} incorporation and reticulocyte values. When the protein-depleted animals were given anemic plasma, cobalt plasma, anemic plasma extract, or cobaltous ion, their erythropoietic rate was elevated to the normal range. Saline had no observable effect. However, as might have been expected, normal plasma elevated both the reticulocyte count and the Fe^{59} incorporation to about one half the extent shown by the anemic plasma.

It is our belief that the reduced rate of erythropoiesis in the protein depleted animals is, in part, secondary to the reduced production of erythropoietin.

The mechanism of a reduced plasma titer of erythropoietin in the protein-depleted rats is not completely clear. One possible explanation, based on our earlier findings on the control of erythropoiesis, is that the metabolic rate of the amino acid-deficient animal is reduced, since it is not making protein at [illegible]

[illegible]

development and growth of red cells.

Erythropoiesis in the Embryo[29]

As previously stated, transfusion-induced polycythemia in mice reduces erythropoiesis to zero as judged by the number of reticulocytes in the peripheral blood, the incorporation of Fe^{59} into the red cells, and the histological examination of blood-forming tissue. This observation suggests that the hormone controlling red cell production is reduced drastically or is absent in mice after the polycythemia is established.

Whether erythropoiesis would be altered in the fetuses of mice in which a transfusion-induced polycythemia was established prior to mating and maintained throughout pregnancy was of interest. To obtain information about this, young adult CF No. 1 female mice were made polycythemic (hematocrits maintained between 60 and 75) by transfusing them with washed homologous red cells. About 6 days later, the animals were found to be reticulocyte-free. They were then mated, and the polycythemia was maintained by repeated red cell transfusion. The polycythemic mothers remained reticulocyte-free throughout gestation. Normal mice of the same strain were mated and studied hematologically throughout their pregnancy. One more control group was used. These mice were mated and then given

mothers and fetuses

At intervals during pregnancy and at term, polycythemic, iron-injected, and normal mothers were sacrificed, and hematocrits and reticulocyte determinations were made routinely on both mother and offspring Spontaneous delivery was allowed to occur in other mice, and similar determinations were made after birth Red blood cell counts and hemoglobin determinations were made on individual fetuses

The hematological studies show that the fetus or the newborn baby mouse born of a polycythemic or Imferon-injected mother had higher hemoglobin, red cell, and hematocrit values than those of the "normal" mother (TABLE 7)

TABLE 7
HEMATOLOGICAL VALUES OF NORMAL, POLYCYTHEMIC, AND IMFERON INJECTED CF No 1 MOTHERS AND THEIR OFFSPRING

	Offspring			Mothers at term		
	Normal	Polycythemic	Imferon injected*	Normal	Polycythemic	Imferon injected*
Hemoglobin (gm)	10	16 4	15 4	11	26	13 8
Red blood cells (cu mm × 10⁶)	3 8	4 6	4 1	6 5	14 0	<9 6
Hematocrit (percentage)	36	51	52	36	65	<50
Reticulocytes (percentage)	51	50	60	3 2	0	4 0

* Total dose, 5 5 mg, intramuscularly

It thus appears that the embryo of a transfusion-induced polycythemic mouse has the capacity to initiate and maintain erythropoiesis, even though no erythropoietin is available to it from the maternal circulation Either no erythropoietin or an insignificant quantity passes from the fetal to the maternal circulation This is based on the finding that no reticulocytes were present in the maternal circulation, an indication that erythropoiesis had not been initiated No evidence of erythropoiesis was found in the hematopoietic tissue taken from the polycythemic mothers that were examined at several intervals during pregnancy or at term

It is true that both CF No 1 mothers and offspring at term are sufficiently iron-deficient to account for the "anemia" The transfused red cells provided enough available iron in the polycythemic mice to produce the higher fetal values, which perhaps should be interpreted as normal values since the control (Imferon) iron-injected mothers likewise gave birth to mice with higher hemoglobin, red cell, and hematocrit values

* Imferon, product of the Lakeside Laboratories, Inc, Milwaukee, Wis

Studies of the Site of Production of Erythropoietin

After the observation that the administration of cobaltous ion or a single massive hemorrhage increases the plasma erythropoietin titer in rats and rabbits within 12 hours,[30] it became possible to study the effect of such procedures as nephrectomy, evisceration, and hepatectomy upon erythropoiesis. In addition, the rapid increase in plasma erythropoietin titer following exposure to low O_2 has appreciably aided us in our efforts to locate the site of production of erythropoietin.[31]

TABLE 8
THE EFFECT OF PLASMA OBTAINED FROM RATS THAT HAD BEEN SUBJECTED TO ORGAN EXCISION AND THEN Co^{++} STIMULATION UPON THE INCORPORATION OF Fe^{59} INTO THE RED BLOOD CELLS OF STARVED RECIPIENTS

Organ removed from donor prior to Co^{++} injection*	Stimulus	Time of removal of blood from donor after Co^{++} injection (hours)	Assay of donor plasma in starved rats using percentage Fe^{59} incorporation, response of recipient to plasma
None	None	12	3 7 (0 4)†
None	Cobalt (250 μM/kg)	12	14 4 (1 5)
Adrenals and gonads	Cobalt	12	15 1 (0 9)
Ninety per cent of the liver	Cobalt	12	12 4 (0 4)
Stomach, intestines, spleen, pancreas	Cobalt	12	11 7 (1 2)
Kidneys	Cobalt	12	4 5 (0 7)
None	None	12	4 8 (0 6)
None	Cobalt	12	16 5‡
Thymus	Cobalt	12	16 3 (1 6)

* Immediately following surgery, the rats (Sprague-Dawley, 350 gm.) were injected subcutaneously with cobaltous chloride, and 12 hours later the blood was collected via cardiac puncture. Plasma thus obtained was assayed in starved rats by standard procedures.
† Standard error.
‡ Three rats.

We undertook a study of the effect of extirpation of various organs or combinations of organs on the capacity of animals to produce erythropoietin in response to Co^{++} or bleeding.[32] As shown in TABLE 8, removal of the adrenals, gonads, 90 per cent of the liver, stomach, intestines, spleen, pancreas, or thymus does not reduce materially the response when compared with that of normal animals. Nephrectomy abolished the response to Co^{++} or acute massive bleeding, whereas bilateral ligation of the ureters reduced, but did not eliminate, erythropoietin production, even though the uremia (BUN elevation) was comparable in both experimental conditions (TABLE 9). The capacity of animals with bilateral ureter ligation to produce erythro-

poietin in response to cobalt or bleeding is greater at 12 hours than at 24 hours after surgery This indicates either that a break down in erythropoietin *production in the kidney (or elsewhere) is occurring as a result of the toxic* nature of the increasing state of uremia, or that the production of erythropoietin is being reduced as a result of local renal effects, perhaps mechanical in nature

The effect of nephrectomy on the rate of disappearance of erythropoietin from the plasma (FIGURE 7) also has been studied as part of this series of experiments Animals were given injections of cobaltous ion and, at intervals

TABLE 9
THE EFFECT OF PLASMA OBTAINED FROM RATS THAT HAD BEEN SUBJECTED TO NEPHRECTOMY OR LIGATION OF THE URETERS AND THEN Co^{++} STIMULATION UPON THE INCORPORATION OF Fe^{59} INTO THE RED BLOOD CELLS OF STARVED RECIPIENTS

Surgical procedures*	Stimulus	Time of removal of blood from donor after Co^{++} injection (hours)	BUN of plasma†	Assay of donor plasma in recipient starved rats using percentage Fe^{59} incorporation Response of recipient to plasma preparation
None	None	—	—	3 3 (0 2)‡
None	Cobalt (167 μM/kg)	12	22	6 6 (1 1)
Nephrectomy	Cobalt	12	99	3 3 (0 5)
Ligation of the ureters	Cobalt	12	95	9 8 (0 1)

* Immediately following surgery, the rats (Sprague-Dawley, 350 gm) were injected subcutaneously with cobaltous chloride, and 12 hours later the blood was collected via cardiac puncture The plasma thus obtained was assayed in starved rats by standard procedures

† Blood urea nitrogen

‡ Standard error

thereafter, nephrectomy was performed and observations were made to determine the rate of fall-off of erythropoietin titer It is evident that bilateral nephrectomy at 4 hours after the injection of cobalt prevented the further rise of titer The parallel fall-off curves are an indication that the loss of activity from the plasma is not significantly different from that seen in the normal animals that had been given a single dose of cobalt Unilateral nephrectomy did not interfere with the normal production of erythropoietin in these rats These data would seem to lend support to the idea that the hormone is elaborated solely by the kidney after cobalt stimulation

In yet another experiment, rabbits were bled by a standard method (hematocrit about 25) and then subjected to nephrectomy or related procedures Twelve hours after surgery the animals were exsanguinated and the plasma was assayed for erythropoietin in polycythemic mice As shown in

Studies of the Site of Production of Erythropoietin

After the observation that the administration of cobaltous ion or a single massive hemorrhage increases the plasma erythropoietin titer in rats and rabbits within 12 hours,[30] it became possible to study the effect of such procedures as nephrectomy, evisceration, and hepatectomy upon erythropoiesis. In addition, the rapid increase in plasma erythropoietin titer following exposure to low O_2 has appreciably aided us in our efforts to locate the site of production of erythropoietin.[31]

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Organ removed from donor prior to Co^{++} injection*	Stimulus	Time of removal of blood from donor after Co^{++} injection (hours)	Assay of donor plasma in starved rats using percentage Fe^{59} incorporation, response of recipient to plasma
None	None	12	3.7 (0.4)†
None	Cobalt (250 µM/kg)	12	14.4 (1.5)
Adrenals and gonads	Cobalt	12	15.1 (0.9)
Ninety per cent of the liver	Cobalt	12	12.4 (0.4)
Stomach, intestines, spleen, pancreas	Cobalt	12	11.7 (1.2)
Kidneys	Cobalt	12	4.5 (0.7)
None	None	12	4.8 (0.6)
None	Cobalt	12	16.5‡
Thymus	Cobalt	12	16.3 (1.6)

* Immediately following surgery, the rats (Sprague-Dawley, 350 gm.) were injected subcutaneously with cobaltous chloride, and 12 hours later the blood was collected via cardiac puncture. Plasma thus obtained was assayed in starved rats by standard procedures.
† Standard error.
‡ Three rats.

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FIGURE 8, bilateral nephrectomy reduced the erythropoietin titer to the normal range.

Because of a report[33] that nephrectomized rats responded to hypoxic hypoxia by the production of erythropoietin, we reinvestigated this question. Although it seemed highly improbable, the possibility existed that response to the low oxygen stimulus was qualitatively different from that induced by hemorrhage. The results, summarized in TABLE 10, demonstrate quite

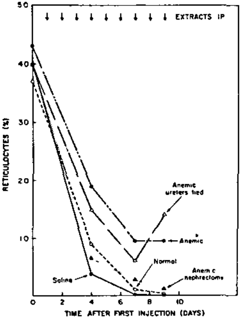
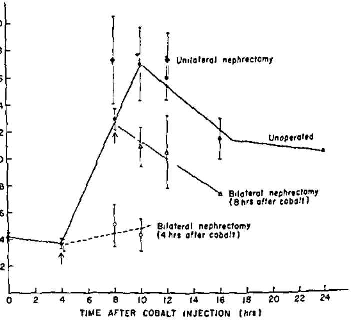


FIGURE 8 The effect of bilateral nephrectomy or ligation of the ureters on the capacity of anemic rabbits to maintain a high erythropoietin plasma titer. The effect of the injection of plasma extract prepared from the plasma of anemic rabbits subjected to bilateral nephrectomy or bilateral ureter ligation upon the reticulocyte values of the peripheral blood of mice with transfusion induced polycythemia as compared with the effect of plasma extract prepared from anemic plasma and normal plasma. Saline was used as an additional negative control.

clearly that nephrectomy, in our hands, abolished completely the response to low oxygen atmosphere (simulated altitude, 21,500 feet), whether at 1 or ½ atmosphere. This is true whether the plasma was assayed in starved or hypophysectomized rats.

The whole problem has several important aspects that must be considered

and polycythemic rat, or the polycythemic mouse), or whether the response



are given

TABLE 10

EFFECT OF PLASMA FROM RATS EXPOSED TO A SIMULATED ALTITUDE OF 21,500 FEET ON THE INCORPORATION OF Fe^{59} IN THE RED BLOOD CELLS OF STARVED RATS AND ON THE RETICULOCYTE VALUES OF TRANSFUSION-INDUCED POLYCYTHEMIC MICE

Time in low O_2 (hours)	Percentage Fe^{59} injection in starved rats*			Percentage reticulocytes in polycythemic mice†		
	Normal unoperated	Nephrectomy	Ureters ligated	Normal unoperated	Nephrectomy	Ureters ligated
8	11 5	4 3	9 8	0 9	0 183	0 7
16	8 6	4 5	9 6	1 7	0 114	1 38
24	11 7	4 7	14 6	1 6	0 006	1 26
None	4 6	—	—	0 001	—	—

* Two injections of 2 ml each, 5 rats per group

† Four injections of 0 5 ml each, 10 mice per group

polycythemic mice and in starved and hypophysectomized rats The results are given in TABLE 10 It is evident that the plasma of normal rats and of

the nephrectomized rats exposed to the low O_2 atmosphere for 8, 16, or 24 hours had no demonstrable erythropoietic activity when assayed in starved or hypophysectomized rats, whereas in the polycythemic mice reticulocyte values reached 0.183, 0 114, and 0 006 per cent, respectively In other comparable experiments, using hypoxic anoxia or anemic anoxia as the stimulus, we have occasionally found that the plasma of nephrectomized animals, when

poietin is made in a site or sites other than the kidney, but that production is quickly poisoned by the toxic state induced by nephrectomy, (2) that the bulk of the erythropoietin(s) is made in the kidney, but that with a severe stimulus some other site can respond feebly, or (3) that this slight response is nonspecific and insignificant?

It must also be considered that the kidney is only one important link in the formation of erythropoietin [32] It seems unlikely that an organ or tissue other than the kidney might produce an inactive precursor in response to cobaltous ion or anemia that is activated in the kidney, because the routine assay procedures employ animals with intact kidneys It is also possible that a precursor originating in some tissue other than the kidney can be activated only by an anoxic kidney We have not yet studied this situation Still another possibility is that the kidney makes an inactive precursor that is activated elsewhere in the body If this were the case, then animals with bilateral nephrectomy would not be expected to respond to cobaltous ion, bleeding, phenylhydrazine, or other stimuli unless another secondary site of production existed

For the reasons stated above, it would be premature at this time to correlate these findings with the frequently reported relationship of renal disease to anemia If further work in this field makes quantitative measurement of erythropoietin activity possible, we shall be in a much better position to determine whether the kidney is the sole producer of erythropoietin(s) or a co-producer.

Erythropoietin in Human Plasma

Recently a number of investigators have turned their attention to the significance of erythropoietin in the human being Contopoulos and his co-workers[35] have demonstrated a plasma factor in the blood of patients with polycythemia vera that is capable of stimulating erythropoiesis in the rat Linman and Bethell[36] have demonstrated an increase in erythropoiesis in the normal rat by injections of extracts of plasma from patients with polycythemia vera and secondary polycythemia Piliero and his co-workers[37]

is measured in terms of the uptake of Fe^{59} or changes in blood volume, the number of reticulocytes, or the histological character of the bone marrow. We cannot state with certainty that a negative response obtained by the use of any of these methods of assay indicates the absence of erythropoietin(s) from the plasma or plasma extracts that are being investigated. It is possible that, after nephrectomy, the donor animals fail to respond to the stimuli (cobaltous ion, bleeding, or hypoxic anoxia) by an increased production, but may produce a normal or a reduced amount of erythropoietin. In either event, attempts to find evidence of activity by testing with whole plasma would fail. In one experiment on rabbits, we used extracts representing threefold plasma concentrations and obtained no evidence of increased erythropoietin activity in the plasma of nephrectomized animals that had been subjected to bleeding or cobaltous ion.[32]

Another possibility has been explored in an attempt to explain the negative results we obtained after nephrectomy. It might be assumed that bilateral

tion. Therefore, it may be that the nephrectomized animal is relatively polycythemic and that the oxygen supply to the animal as a whole is increased. If this is true, it might be expected that the production of erythropoietin would be reduced. To test this, we have bled animals, reducing their hematocrits to about 25 per cent. Under these experimental conditions, the erythropoietin titer was high. When bilateral nephrectomy was performed in these animals and the anemia was maintained, the erythropoietin titer fell to control levels. In addition, we have surgically removed other tissues or combinations of tissues (90 per cent of the liver or evisceration) that might be expected to produce a "relative polycythemia" comparable with that produced by bilateral nephrectomy. These animals responded to cobaltous ion, bleeding, or hypoxia with an elevation of erythropoietin.

It is quite possible that the toxic condition resulting from bilateral nephrectomy is qualitatively different from the uremia that results when both ureters are ligated. There is the likelihood that the "toxic" reaction following bilateral nephrectomy, under the experimental conditions described in this paper, interferes with the action of the different stimuli at some site other than the kidney. In addition, the site of erythropoietin formation may be affected directly by the toxic agent(s). Recently, Erslev[34] has published data on experiments on nephrectomized rabbits from which he concludes that the site of erythropoietin is not in the kidney and that the anemia of nephrectomized animals is related to the metabolic changes associated with the uremia.

In a series of experiments we have completed only recently,[25] groups of nephrectomized rats were exposed to hypoxic conditions (simulated altitude, 21,500 feet) for periods of 8, 16, or 24 hours, respectively. As in previous experiments, controls consisted of groups of rats with bilateral ureter ligation and normal rats similarly exposed to hypoxic conditions. Plasma was obtained, and assay was conducted simultaneously in transfusion-induced

the normal range by transfusion *In other words, the normal mechanism* (anemic anoxia) exists, and the normal response (increased erythropoietin production) occurs by shutting off the stimulus (transfusion), erythropoietin production then falls.

In other patients, no response to anemic anoxia may occur as measured by plasma or urine erythropoietin titer In most cases, this can be explained on the basis of such factors as nutrition, infection, and renal disease. In other instances, an anemia may exist in response to decreased metabolism (hypothyroidism[40] or hypophysectomy[20]) in which no anoxic stimulus exists and production and utilization of erythropoietin are in equilibrium

Since the erythropoietin titer in normal plasma cannot be measured with any degree of accuracy, and the elevated plasma and urine titers that occur *in response to various anoxic stimuli cannot be quantitated with any accuracy*, it is impossible at present to establish the renal threshold above which this hormone is excreted It would be interesting indeed to establish the threshold above which excretion would occur and measure accurately the total urinary output under such circumstances, relating it to the plasma levels

Summary

The erythropoietic activity of plasma from anemic animals and human beings can be determined by studying the rate of incorporation of Fe^{59} into the red cells of recipient *normal* rats *Hypophysectomy of rats reduces erythropoiesis to a minimum within 1 to 3 weeks The reduction is less and* occurs more slowly in immature rats than in young adult rats *Hypophysectomized* rats are sensitive to anemic plasma, especially during the period (between 1 and 3 weeks) when erythropoiesis is at its minimum The

cythemia in rats and mice, acute starvation in rats, and hyperoxia in rats, all rapidly reduce erythropoiesis Experimental animals in these conditions are sensitive in their response to the injection of erythropoietin

The rate of erythropoiesis, as determined by the quantity of circulating erythropoietin, depends on the relationship of oxygen supply to oxygen demand in the tissue rather than on either factor acting alone

Injection of cobaltous chloride increases the erythropoietin titer in the plasma of rats and rabbits within 10 to 12 hours

After bilateral nephrectomy, the administration of cobaltous ion, acute hemorrhage, or the induction of hypoxic anoxia fails to elevate plasma erythropoiet [illegible]
nate the eryt [illegible]
stomach, int [illegible]
increase in plasma erythropoietin does occur in response to cobalt or bleeding

Erythropoietin titer was assayed in these experiments by measuring the effect of the various plasma samples on the incorporation of Fe^{59} *into the red* cells of recipient starved or hypophysectomized rats. Plasma from nephrec-

have found an erythrocyte-stimulating factor in the plasma of patients with Cooley's anemia and sickle-cell anemia. Their report also reviews previous work with human plasma by other investigators.

We have described[8] the demonstration of erythropoietin in the extracts of heat-denatured plasma obtained from some anemic patients Using the incorporation of Fe^{59} into erythrocytes of hypophysectomized or starved male Sprague-Dawley rats, erythropoietin has been shown to be present in plasma from normal human beings However, a tenfold concentration is necessary for such a demonstration when plasma extracts are prepared by heat denaturation or perchloric acid precipitation. Without concentrating the plasma extracts, erythropoietin has been demonstrated in heat-denatured extracts from twenty anemic subjects Erythropoietin has been found to be present in the urine in some, but not all, of the patients whose plasma demonstrated high titer [33] Hypoplastic anemia in five patients could not be attributed to a decreased plasma erythropoietin concentration. The high erythropoietin concentrations in the blood and urine of one patient with hypoplastic anemia fell promptly following blood transfusion It must be emphasized that failure to demonstrate activity by this method does not necessarily indicate a decreased erythropoietin concentration.

Comments on Production and Utilization of Erythropoietin

If a stimulus such as hypoxic anoxia or anemic anoxia were applied with gradually increasing severity to an animal, one would expect erythropoiesis to increase as erythropoietin production increased. However, it is quite possible that plasma titer of erythropoietin would not be measurably above the normal (by current methods of assay) and, therefore, that no measurable amount would be excreted. In other words, one might consider that utilization paralleled production This concept would explain our observation that assay of plasma from patients with a moderate anemia but with an active marrow, as in patients with hemolytic anemia or pernicious anemia, may reveal little or no erythropoietic activity However, if the stimulus were severe and abrupt, one would expect to find erythropoietin in the plasma and perhaps, in the urine as well, because erythropoiesis could not increase rapidly enough to utilize available erythropoietin Similarly, if the stimulus were increased gradually, one would expect that only as soon as erythropoiesis reached its maximum (calculated to be six times normal) would excess erythropoietin be found in the plasma and, perhaps, in the urine, and then only if the stimulus for and actual production of erythropoietin exceeded utilization

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tomized animals exposed to h
induced polycythemic mice
erythropoietic response was o
in relationship to our suggestion that the kidney may be a site of erythropoietin production

The production, utilization, and excretion of erythropoietin are discussed briefly

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SOME NEW EXPERIMENTAL DATA ON THE ROLE OF TH ADRENALS IN THE REGULATION OF BLOOD EOSINOPHIL

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Introduction

The eosinopenic action of ACTH and glucocorticoids has given ris innumerable publications, especially since the introduction by Thorn *et a* their test of adrenocortical activity based on the eosinopenic response to AC

Nevertheless, there still remain many unresolved problems concer the mechanism of the effect of glucocorticoids upon the eosinophils, as as the role played by these hormones in the stress eosinopenias.

To explain the disappearance of these cells in the blood stream, four p bilities are considered (1) the destruction of these cells in the blood ve (2) their temporary migration into different organs; (3) their phagocy by the reticuloendothelial cells, and (4) a blocking of their production ripening in the bone marrow.

This problem has been treated extensively by Gordon.[2] Recently I, published a review[2a] on the hormonal regulation of the blood eosinophi

The subject of this paper is the possibility of the existence of hur mediators in the production of eosinopenia induced by cortisone or by va stressing conditions The intervention of such mediators would explai absence of any direct action of cortisone upon eosinophils *in vitro*

These hypothetical substances should act on the cells in a much sh time than cortisone In fact, Tanos and his group[3] have reported that l serum obtained from cortisone-injected rats at the peak of their eosino response (3 to 4 hours after the injection), itself induced a very rapid eo penia (in 10 to 30 min) when injected into other rats.

A priori, two categories of mediators are to be considered: (1) catab produced by degradation of cortisone itself, and (2) catabolites issuing tissues that have been injured by cortisone.

The first hypothesis seems to be eliminated by the experiments of S (reported in Gordon[2]), who was unable to produce eosinopenia with a the steroids isolated after liver perfusion with cortisone

Hays and Zaratzian[6] suggest that even the eosinopenia produce

cortisone is due to histamine, for they succeed in preventing this effect by antihistaminic drugs. However, other experimental data do not support the hypothesis of an autonomic eosinopenic action of histamine. Thus Halpern and Benos[8] find that, in the rat, histamine loses its effect upon the eosinophils after hypophysectomy. On the other hand, in the same species the interval between the injection and the peak of the eosinopenia is generally as long with histamine as with cortisone (Aschkenasy*).

Furthermore, the fall of the number of blood eosinophils produced by histamine is perhaps only the consequence of an accumulation of these cells in the injected areas, as noticed by Archer[9] in the horse after intradermal or intramedullary injections of this compound. However, Speirs[10] did not find any focal accumulation of eosinophils after intraperitoneal injection.

Moreover, Code *et al.*[11] and Kelemen and Bikich[12] show that administration of cortisone not only does not increase, but diminishes the blood amount of histamine, as well as the number of eosinophils. Perhaps, this effect is explained by the cortisone-induced inhibition of the formation of histamine from histidine.[13]

According to Archer,[14] histamine represents the normal stimulating agent of the eosinopoiesis, and the cortisonic eosinopenia results from the blocking of maturation of eosinophils following the suppression of this agent.

Thus the role of histamine has been interpreted in very divergent ways: some authors have attributed to this compound an eosinopenic potency, either direct or indirect, implicating a stimulation of the adrenal cortex, whereas, on the contrary, other workers have pointed out an eosinotropic or even eosinopoietic function of this substance.

Nevertheless, histamine apparently is not to be included among the tissular catabolites mobilized by cortisone, which inhibits its formation.

We shall consider the case of other tissular metabolites that may play a role in the eosinopenia observed after administration of cortisone and in the so-called alarm reactions.

At present it is generally agreed that the action of the glucocorticoids is not limited to the deamination of some amino acids and to the subsequent production of ketonic acids, glucose, and glycogen,[15] but that this action includes also the labilization and mobilization in the blood stream of entire molecules of proteins[16] and their disintegration into amino acids.[17]

These concepts would explain the decrease in the amount of free amino acids in the blood after adrenalectomy,[18] as well as the increase of this amount after injection of ACTH,[19] cortisone[20, 21] and hydrocortisone.[22]

On the other hand, the deamination could be one of the reasons for the increase of blood pyruvic acid which is recorded after injection of the same hormones[23-27] and also in various alarm reactions.[28, 29]

We had to consider the question of whether the eosinopenia should be attributed to the noxious action of some of these intermediary products of the protein metabolism, products delivered into the blood stream by glucocorticoids: either these compounds destroy directly the eosinophils or diminish

* Unpublished

their resistance to the phagocytic activity of the reticuloendothelial cells or enhance the macrophagic potency of these cells.

If this hypothesis were justified, the responsible compounds should keep their eosinopenic potency even in adrenalectomized animals and perhaps also produce their effect in a shorter interval than cortisone does

Furthermore, the simultaneous administration of these substances and cortisone should amplify the eosinopenic response in these same adrenalectomized animals

Finally, every tissular disintegration provokes not only the discharge of organic catabolites, but also the release of intracellular potassium Therefore, one could assume that this electrolyte itself might be one of the responsible factors of the cortisone eosinopenia

All of these considerations justify the manner in which our experiments have been done

Role of Serum Proteins and of Some Organic Metabolites in Cortisone Eosinopenia

Comparison of Intact and Adrenalectomized Rats

In our first study we have compared in intact and in adrenalectomized rats the action exercised on eosinophils by the following substances: bovine serum albumin, human γ-globulin, a mixture of amino acids following approximately the proportions existing in serum proteins;[20] 14 amino acids injected separately, urea, and several carbohydrate compounds, some of which are known to be increased in the blood during stresses or after injection of cortisone sodium pyruvate, lactate, oxalacetate, α-ketoglutarate, glucose, and citrate

All these substances were injected intraperitoneally into adult white rats in doses of 0 025 gm , 0 050 gm or 0 100 gm., the first time before and the second time after adrenalectomy Each rat received 2 series of 3 different products, the injections being administered at 5-day intervals

Nevertheless some rats did not receive certain substances, either before or after adrenalectomy, which explains the unequal number of animals in

the day for each animal

All adrenalectomized rats received drinking water containing 1 per cent NaCl

To preclude the possibility of any error due to the persistence of accessory adrenal tissue, which is very frequent in white rats, we kept for our final

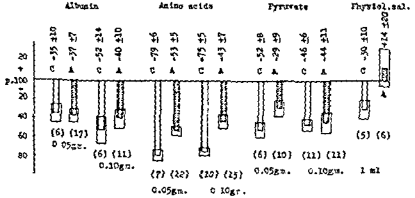


FIGURE 1 Percentage changes (mean values ± standard error of the mean) in the number of blood eosinophils 4 hours after intraperitoneal injections of serum albumin, of a *mixture of amino acids, or of sodium pyruvate (0.05 gm or 0.10 gm) in controls (C)* and in adrenalectomized rats (A) Comparison with the effects of physiological saline (1 ml) Figures in parentheses represent the number of rats in each group

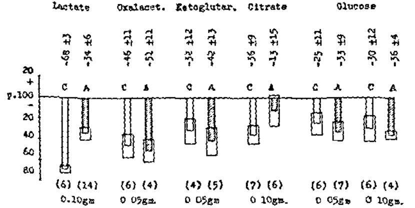


FIGURE 2 Percentage changes (± S E M) in the number of blood eosinophils 4 hours after intraperitoneal injections of sodium lactate, oxalacetate, α ketoglutarate, citrate, or glucose in intact (C) and adrenalectomized (A) rats Figures in parentheses represent the number of rats in each group

at necropsy of a hypertrophied thymus and apparent absence of any formation that could be suspected to be an accessory adrenal, with histological verification in dubious cases

Results FIGURES 1 and 2 show that albumin and the amino acid mixture, as well as the carbohydrate compounds, induce significant falls in the number of blood eosinophils in intact rats The same is true with urea, which is not included in our figures

All of these substances except urea and sodium citrate also promote eosin penia in adrenalectomized rats It is true that in the adrenalectomiz animals the eosinopenic response is often relatively moderate, neverthele the results are significantly different from those recorded after intraperitone injection of 1 ml of physiological saline which, on the contrary, provoke slight increase of eosinophils in the operated rats

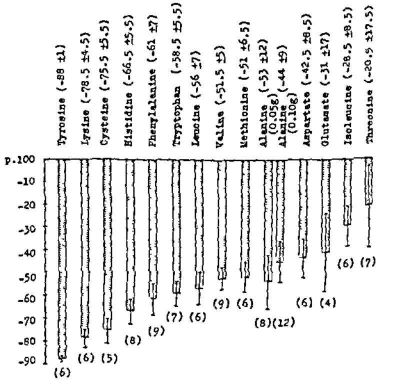


FIGURE 3 Percentage changes (± S E M) in the number of blood eosinophils af intraperitoneal injection of various amino acids in intact rats

In order to analyze the action of the amino acids mixture more closely, compared the isolated effect of various amino acids (FIGURES 3 and

Some amino acids (tryptophan, leucine and isoleucine, threonine, a sodium glutamate) lose almost all of their potency, but several others s provoke a significant eosinopenia Generally, the latter is less strong aft the operation than before (tyrosine, histidine, phenylalanine, methionir

and sodium aspartate) Only valine is approximately as effective in adrenalectomized rats as it is in controls

It is noticeable that not only most of the amino acids but also all the other compounds tested thus far are (except for ketoglutarate and glucose) less active in adrenalectomized rats than in intact rats

These results suggest that almost all of these metabolites act at the same

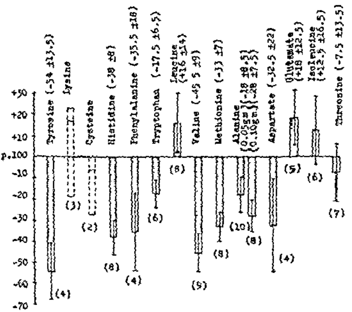


FIGURE 4 Percentage changes (±S E M) in the number of blood eosinophils after intraperitoneal injection of various amino acids in adrenalectomized rats Only individual changes of 3 and 2 rats are given for lysine and cysteine because of the death of the other rats of these 2 groups

time in an autonomic way, independently of the adrenals, and also by the intermediary of these glands in apparently inducing a discharge of ACTH

Like albumin, γ-globulin provokes eosinopenia in adrenalectomized rats, as well as in controls (FIGURE 5) This fact permits a new interpretation of the eosinopenias observed in various diseases with hypergammaglobulinemia

Indeed, in opposition to albumin, γ-globulin not only does not increase in the blood after injection of glucocorticoids, but even diminishes in most cases, conversely, adrenalectomy is followed by an augmentation of this globulin[31, 32]

Therefore, it is difficult to believe that the hypergammaglobulinemia recorded in most infections, intoxications, or neoplastic and nutritional diseases results from a hyperactivity of the adrenal cortex

It is more probable that the increase of γ-globulin is due to a direct ir tion of the reticulum of globulin-producing organs (chiefly bone marr the responsible agents of this irritation being either of microbial, toxi metabolic origin

In any case, this hyperglobulinemia should play a certain role in prom the eosinopenia observed in these pathological states

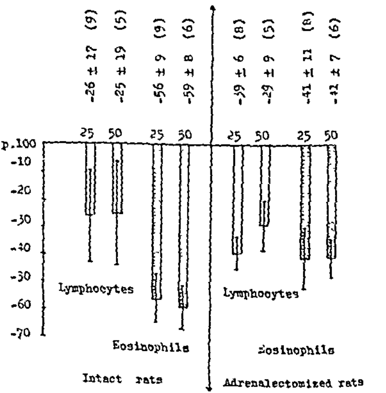


FIGURE 5. Percentage changes (±S.E.M.) in the number of blood lymphocytes eosinophils after intraperitoneal injection of γ-globulin (25 or 50 mg.) in intact and adrenalectomized rats. Numbers of rats are given in parentheses

superiority of the falls of the eosinophil number after injection of γ-globu in intact controls in comparison with the falls recorded in adrenalectomi rats.

Thus the hyperactivity of adrenals noticed in diseases with hypergl linemia would be not the cause but rather the consequence of this lat abnormality.

Moreover, there is some reason to believe that in these diseases the incre

of γ-globulin is not the only humoral factor responsible of the eosinopenia Perhaps a certain role is played by polypeptides and free amino acids, as well as by pyruvic acid, which are all increased in the blood and which likewise

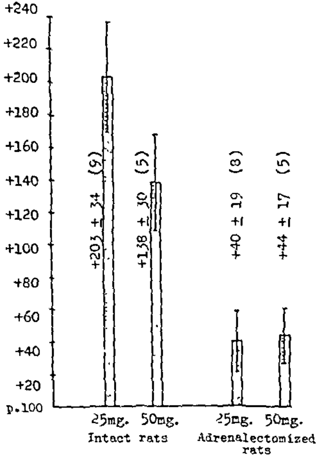
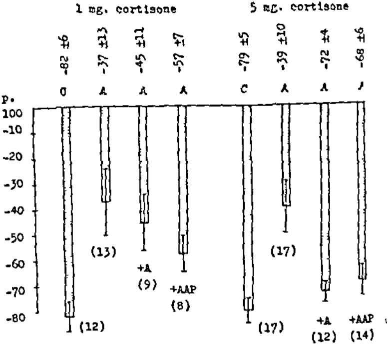


Figure 6. [illegible]

simultaneously act directly and indirectly through stimulation of the adrenal cortex

As FIGURE 5 shows, the injection of γ-globulin provokes not only an eosinopenia, but also a drop of the level of lymphocytes, this drop is almost identical before and after adrenalectomy

On the contrary, the number of neutrophils increases considerably after the same injection (FIGURE 6) However, this increase is recorded chiefly in control rats (from 100 to 230 per cent), whereas it is only about 40 per cent in adrenalectomized ones



... percentage ... injection ... pyruvate

... the eosinopenia, ... ta- ... hat ... the

adrenals and without any mediation of these glands

In any case, the direct responsibility of the adrenals in the hematological disturbances of these pathological states does not appear to be as prevailing as one used to assume until now

Reinforcement of the Eosinopenic Action of Cortisone by Certain Organic Metabolites

Intraperitoneal injection of cortisone (1 or 5 mg) induces eosinopenia which is less [illegible] tant in adrenalectomized rats receiving saline drinking water th[illegible] ts (FIGURE 7)

Such a paradoxical diminution of the effect of cortisone after adrenalectomy has already been reported by Henry *et al* in the dog[13] These authors have succeeded in amplifying the response of the operated animals by simultaneous injection of epinephrine.

Thevathasan and Gordon (Gordon[2]) have also obtained a potentiation by epinephrine of the eosinopenic action of an adrenocortical extract in rats that had been deprived of both spleen and adrenals

On the contrary, Essellier *et al*[14] did not find any reinforcing effect of epinephrine upon the action of cortisone in adrenalectomized dogs

Our own experiments show that enhancement of cortisonic eosinopenia in adrenalectomized rats also can be obtained by means other than injection of epinephrine (which we did not try). for example, by adding to cortisone a preparation of amino acids or better, a mixture of amino acids, albumin, and pyruvate (AAP of FIGURE 7) This latter mixture was active even when the amino acids alone were devoid of any effect (as was the case with 1 mg cortisone).

On the contrary, the same adrenalectomized rats did not amplify significantly their eosinopenic response to cortisone when they were injected simultaneously with cortisone and 7 5 mg ACTH This fact proves that the animals were free of accessory adrenals and that the metabolites have acted in an extra-adrenal way and not only by stimulating accessory adrenals by the intermediary of hypophyseal discharge of ACTH

Hourly Evolution of the Eosinopenia Provoked by Amino Acids and Pyruvate

All the preceding results make it possible to infer that various substances, he blood levels of which increase after administration of cortisone or during larm reactions, induce eosinopenias without necessarily affecting the drenals.

Nevertheless, as the example of amino acids and pyruvate suggests (FIGURE 8), the maximum of the eosinopenia produced by these substances is recorded only after 3 or 4 hours and therefore is not in advance of the eosinopenia following an injection of cortisone. Thus it becomes difficult to suppose that cortisone acts upon the eosinophils only by the intermediary of amino acids and keto acids released into the circulation by tissues injured by cortisone

It is more probable that the cortisone eosinopenia is a complex phenomenon that, in addition to the specific and still mysterious action of cortisone itself, includes an intervention of various tissular catabolites, these catabolites provoke a discharge of ACTH and also act independently upon the eosinophils, thus contributing to amplify and to prolong the specific effect of cortisone

Role of Electrolytes in Cortisone Eosinopenia

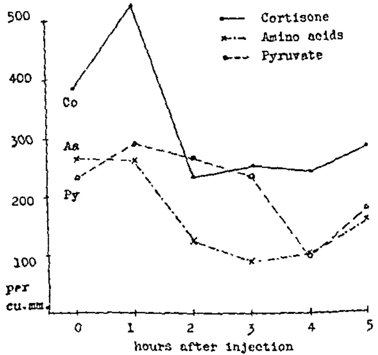
As FIGURE 9 shows, intraperitoneal injection of potassium chloride induces a [illegible]

a

o

of 0 100 gm. had provoked a shock followed by death in most adrenalecto-mized animals

Therefore the possible discharge of potassium from stressed tissues acts only by stimulating the hypophysoadrenocortical system because this electrolyte is deprived of any effect on the eosinophils in the absence of adrenals



In opposition to potassium chloride, sodium chloride injected in do- 0 050 gm and 0 10 gm indures eosinopenia in adrenalectomized rats, as as in intact ones On the contrary, a very dilute solution of NaCl (ph logical saline) is effective only in intact rats (probably only because c stress of the injection), whereas it provokes an increase of the eosinoj after adrenalectomy (FIGURE 9)

The oral absorption of 1 per cent solution of NaCl even inhibits the ec penic response to cortisone, as indicated by the comparison of the ec

because the intact animals can control the blood levels and the excretion of the electrolytes by an adaptation of their adrenocortical activity

The decrease of cortisone eosinopenia after adrenalectomy, as reported by Henry and his group in dogs and observed also by ourselves in rats, is entirely suppressed if the operated animals are given tap water in place of saline This effect is more significant with 5 mg than with 1 mg cortisone [25]

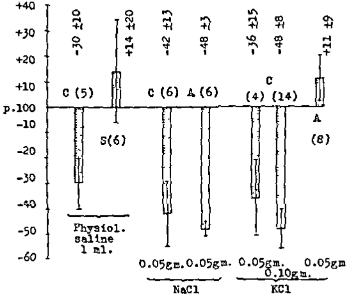


FIGURE 9 Percentage changes (± S E M) in the number of blood eosinophils after intraperitoneal injections of physiological saline and of concentrated solutions of NaCl or KCl in intact (C) and adrenalectomized rats (A) Number of rats in each group is given in parentheses

We have reported above that a similar amplification of the cortisone eosinopenia has been obtained by us in adrenalectomized rats receiving NaCl, by injection of amino acids, or by simultaneous injection of amino acids, albumin, and pyruvate

We were greatly surprised to discover that injections of desoxycorticosterone (glucoside) given to adrenalectomized rats receiving tap water did not diminish the eosinopenic response to cortisone This response remained equally strong (approximately 60 per cent) whether the injection of cortisone was performed in the absence of desoxycorticosterone or after a previous treatment with this hormone (1 mg per day) during 5 days (FIGURE 10) Simultaneous injection of both hormones gave the same result as injection of cortisone alone.

These results appear to contradict the hypothesis that desoxycorticosterone is antagonistic to cortisone in the regulation of blood eosinophilia More-

over, they indicate that there is no similarity of action between administr of sodium and injections of desoxycorticosterone

The inhibitory effect of NaCl on cortisone-induced eosinopenia leads consider the possibility that the organic metabolites tested in our prec experiments would also provoke more pronounced eosinopenias in adrei tomized rats if these rats had received tap water instead of 1 per cent

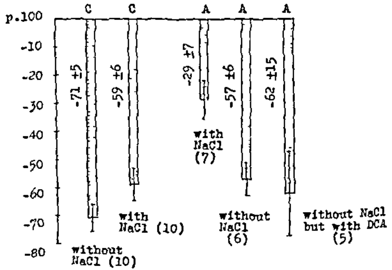


FIGURE 10 *Reduction of the eosinopenic response (percentage changes* ±S E M

In this case, the participation of adrenals in the eosinopenic respons intact rats to these metabolites would be even less than we have admit Experiments are now in progress to clarify this problem.

CONCLUSION

(2) Furthermore, our experiments suggest a new explanation of cer eosinopenias recorded in many infectious, neoplastic, and nutritional dise which are accompanied by an increase of γ-globulins. Until now t eosinopenias have been attributed to a stimulation of the adrenal cor but such a stimulation should induce a decrease rather than an increas γ-globulins.

Therefore it seems more probable that the hyper-γ-globulinemia found in these diseases is independent of the adrenals and is itself partly responsible for the reduction of the number of blood eosinophils

Furthermore, the action of the globulins may coincide with that of other nitrogenous and carbohydrate metabolites which, like albumin and globulin, act upon the eosinophils at the same time directly and probably also indirectly through the hypophysoadrenocortical system

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HEMOLYTIC MECHANISMS*

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Much has been learned about hemolytic mechanisms in the decade since ie of us[1] contributed a paper to a previous monograph on the same topic t that time the emphasis was chiefly on morphology and pathophysiology, owever, in the last few years the chemical approach has gained increasingly . importance This shift may be recognized throughout the entire field of ematology and, indeed, of medicine in general It was necessary first to escribe diseases and study them morphologically in various ways Beginng about 1920 and extending until recently, the age of clinical investigation ominated the scene, the pathophysiology of disease being its important ature We are now in a chemical era in which numerous new mechanisms e being exposed as the chemical tool etches its certain way into many cells id tissues Perhaps in the future biophysical and even mathematical eras ill come to the forefront, but today's advances are largely chemical, although morphologic renaissance seems even now to be in the making From the andpoint of hemolytic mechanisms, there is room for all methods of study, id all may be helpful in gaining ever deeper insight into events at the llular level

The sudden destruction of erythrocytes within the body, together with the evelopment of severe hemolytic anemia, is one of the most dramatic events clinical medicine. As Crosby has pointed out,[2] this is decompensated emolysis However, hemolysis is a normal, everyday occurrence, approxiately 1 per cent of the red cell mass being destroyed, but also replaced ily This is a quiet and compensated type of hemolysis, so quiet in fact iat the mechanisms by which it occurs are rather difficult to define accutely, in fact more difficult than in abnormal hemolytic states

Normal Hemolysis

In the past, the red cell was considered an inert, amorphous mass of protoasmic material enclosed in a semipermeable sac that became gradually orn out by the wear and tear of the circulation and was finally destroyed, erhaps by lytic substances, perhaps in the spleen It is realized now that e erythrocyte, although nonnucleated, is a living, breathing structure that quires various sources of energy to maintain its metabolic needs [3] Some these sources may be said to be built in or intrinsic, whereas others are erived from the outside milieu Thus, over forty years ago, it was demon-

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strated that red cells could be preserved for lengthy periods if an energy source, namely, glucose, was added to the preserving media [4] In recent years it has been shown that the red cell depends almost exclusively on

lesion)[7] is due to the gradual decline of nonreplicating, "built-in" enzymes, many of which are present in significant quantities in the red cell as it is released from the bone marrow [8] Like a rocket that is launched with a carefully measured amount of fuel sufficient for its journey through space, the erythrocyte propelled from the marrow is replete with numerous metabolic constituents lipid, protein, lipoprotein, carbohydrate, enzymatic, and others, sufficient for its normal life span When these become depleted, the cell dies

The Life of a Red Cell

The new red cell comes out of the marrow thin, biconcave, and somewhat larger than the older erythrocyte It squeezes in and out of capillaries, it is buffeted about by the pulsating circulation, it may stagnate for hours in the sinuses of the spleen or liver In the spleen and in other areas it may come in contact with lytic substances of a low order of potency. lysolecithins, soaps, other tissue lysins [9] The wear and tear hypothesis, although a purely physical one, nevertheless may have some merit although, now that much more is known of the metabolic functions of the erythrocyte, this purely physical description does not tell the entire story In any event, the combined effect of extrinsic forces (mechanical trauma, erythrostasis, lytic substances) in association with reduction in enzymes and other nonreplicating materials leads t[illegible]
svelte, its conca[illegible]
the criteria for [illegible]
thicker than normal, with a diminished diameter and a smaller surface are[illegible]
[illegible] 1 per cent of the cel[illegible]
[illegible], rounded, microcy[illegible]
[illegible] that these cells a[illegible]
pocked with surface breaks [10] Physiologically, the hypotonic fragility increased. Enzymatic studies have shown that phosphatase,[11] cholinesterase,[12] catalase, and glyoxalase[13] decline progressively as the cell grows olde[r] the greatest quantities of these enzymes being present in the reticulocyte There is a decline in potassium and an increase in sodium and phosphorus ion within the cells.[14] A reduction in the high energy phosphate compoun[d] takes place[15] and, at the more fundamental level, a reduction in ionic transfe[r] occurs.[16, 17]

All in all, therefore, as the cell moves around the many miles of capillari[es] in the course of its existence, it "runs out of gas," precisely as does the rock[et]
[illegible] l and the
Destru[illegible]
ed [18] Th[illegible]

role of tissue lysins, including the rather vague lysolecithin, probably has been overemphasized and may well be discounted Tissues have been shown by many investigators to have lytic activity *in vitro*,[19-21] but whether the highly artificial conditions existing in the test tube can be compared with what occurs *in vivo* is highly questionable The presence of fatty materials in increased quantities after a meal has been cited as a possible mechanism for normal lysis,[22] but again this is a moot point

Although it is attractive to envision the spleen as a major site of normal red cell destruction, there is no compelling evidence regarding this point

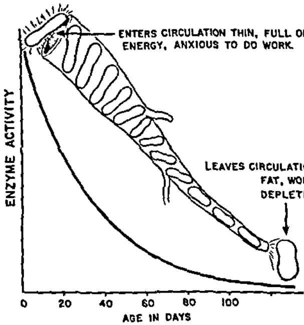


FIGURE 1 Life of a red cell

Indeed, Singer and Weisz[23] could find no difference in red cell life span of dogs before and after splenectomy The peculiar vascular structure of the spleen, the observation that *in vitro* incubation of red cells,[24] and their stagnation in the spleen, result in increased spheroidicity with increased mechanical and osmotic fragility[25] provides only circumstantial evidence of its role in the physiological destruction of blood Curiously enough, the actual *coup de grace* by which the red cell dies is unknown It may be phagocyted by elements of the reticuloendothelial system, although actual evidence for this is lacking, it may be fragmented, it may be hemolyzed suddenly It is tempting to consider that these events take place in the spleen, which has often been listed as the slaughterhouse of the red cell, if not its graveyard as well How-

ever, with the normal spleen removed, hemolysis goes on at approximately normal rates

In summary, then, the life of the red cell may be thought of as follows: it is released from the marrow fresh, svelte, biconcave, and fully primed with active metabolites for its lengthy journeys, which continue for a period of about four months. At the end of that time, the red cell is thick, fat, lacking in metabolic constituents, including certain enzymes and high energy PO_4 donors, and with a deranged ionic composition (FIGURE 1). How the death blow is administered is not known, but that it occurs with remarkable regularity after 120 days is well established

Some Indicators of Normal Hemolysis

More is known of the indicators of hemolysis, both normal and abnormal, than of its mechanisms. It is of considerable interest that, of the approximately 50 ml of (whole) blood hemolyzed daily, only a few milligrams of hemoglobin are found free in the plasma. This indicates that normal hemolysis is almost exclusively extravascular, that is, occurring outside the circulation, presumably within the cells of the reticuloendothelial system. On the other hand, under certain abnormal conditions, hemolysis may be largely intravascular, with the liberation of free hemoglobin directly into the circulating plasma.[26] Indications of hemolysis vary as between these two types of blood destruction. In both, the red cell survival time is shortened, and this event establishes the hemolytic state (TABLE 1)

Table 1
Types of Hemolysis

	R-E or extravascular (speeded up normal)	Intravascular (abnormal)
RBC survival time	+ to +++	+++
Bile pigment output	+ to +++	± to +
Hb in plasma	± (less than 25 mg /100 ml)	50 mg /100 ml up (haptoglobin)
Hb in urine	0	0 to +++
Hemosiderinuria	±	+++
Methemalbumin in plasma	0	+
Fe in plasma	Less than 100 gamma	++
Spherocytosis	Usually present	Usually absent
Reticulocytes	+ to +++	+ to +++

The degree of spherocytosis parallels the abnormality of the osmotic fragility test,[27] the latter having no greater importance as a measurement of hemolytic activity than the morphologic appearance of the red cells. In measuring hypotonic fragility, the older methods of estimating hemolysis in

graphic representation of the red cell thickness variations This[28] (FIGURE 2) is of particular value in the thalassemic syndromes

Mechanical fragility, using glass beads to traumatize the red cells, was first used by one of us to indicate the abnormality of the red cell after exposure to immune body It was then used by other workers as one indication of erythrocytic abnormality but, except possibly as an experimental tool, its value is to be questioned [29]

Shortened red cell survival,[30] increased pyrrole pigment output,[31] hemoglobinemia and, at the cellular level, spherocytosis these are the direct indications of increased hemolysis However, some of the indirect parameters may often be of great importance, particularly in "spotting" a hemolytic state Of these, reticulocytosis is pre-eminent, representing as it does the tendency on the part of the bone marrow to regenerate itself after some of its products,

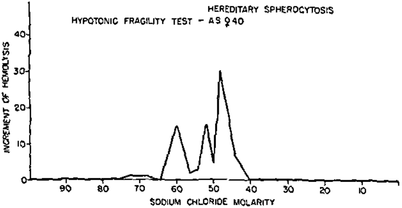


FIGURE 2

the erythrocytes, have been destroyed We have already commented on the importance of persistent reticulocytosis of even mild degree (about 4 to 5 per cent) as indicating the presence of a hemolytic disorder, and also upon the biphasic red cell population seen in active hemolytic disease [32] In the latter phenomenon, there is a striking contrast between the microcytic spherocytes (indicators of hemolysis) and the macrocytic polychromatophilic reticulocytes (indicators of increased erythropoiesis) The bone marrow itself shows an outstanding degree of erythroblastic hyperplasia and a high ratio of nucleated red cells to granulocytes

Hyperhemolysis, whether extravascular or intravascular, may furthermore be subdivided into two main groups those in which the defect is intrinsic to the red cell proper, and those due to extrinsic factors active in undue fashion upon the normal red cell (TABLE 2) Although most of the intrinsic abnormalities of the red cell are under genetic control, others can be acquired All the extrinsic abnormalities may be said to be acquired. Another more occult defect recently has come to the fore Although fundamentally of

TABLE 2

TYPES OF HEMOLYTIC ANEMIA AND HEMOGLOBINURIA

Intrinsic		Extrinsic
Hereditary spherocytosis	Hereditary or genetic	Infections
Thalassemia		Chemical
Sickle cell disease		Immunological
The Hemoglobinopathies		Isohemolysis (Rh, etc)
Hereditary ovalocytosis		Autohemolysis
Pernicious anemia		Hemolytic anemia
Paroxysmal nocturnal hemoglobinuria		Paroxysmal cold hemoglobinuria
March hemoglobinuria(?)		Donath-Landsteiner
		Hyperspleníc
		Others

Occult intrinsic defects requiring extrinsic "challenge"

intrinsic nature, this does not show itself unless, in ...
factor of a specific type enters the ...
in such conditions as primaquine ...
intrinsic and extrinsic types of hem...

INTRINSIC DEFECTS OF THE RED CELLS AS A CAUSE FOR HYPERHEMOLYSIS

Morphologic Aspects

Until recently, the intrinsic defects of the red cells have been classifi[ed] largely on morphologic grounds Although the pendulum has swung f[ar] away from the morphology of the cell to its chemical make-up, study of t[he] appearance of the mature red cell is still of some value. One may distingui[sh] on morphologic grounds alone, several forms of hereditary hemolytic diseas[e] spherocytosis, leptocytosis (target cells), drepanocytosis (sickle cells), oval[o]cytosis, and nonspherocytosis In acquired hemolytic disease, there is t[he] ... -ar ... her ... uri ... n c ... stri ... pecu- ... echa-nisms: perhaps the small spherocyte is vulnerable to the spleen, perhaps the tiny schistocytes seen so commonly in severe thalassemia are also readily destroyed by that organ, perhaps the sickled cells "lock horns" with each other in capillaries Certainly, morphology can tell us nothing as to why the red cells of hereditary nonspherocytosis hemolyze or why those of march hemoglobinuria are rapidly destroyed in goose-stepping soldiers With the increasing development of physiopathological and chemical techniques, our understanding of the hemolytic mechanisms involved in the various intrinsic defects has become greatly increased.

Physiopathological Aspects

Hereditary spherocytosis. In the course of the past twenty years, many studies have gone far to establish the hemolytic mechanism of this disorder

here is an exact correlation between the reaction to hypotonic salt solutions and the thickness or degree of red cell spherocytosis,[34] and further studies have demonstrated that the spherocyte was trapped selectively by the spleen, the degree of trapping depending upon the degree of thickness increase.[35] Red cell life span studies and cross survival experiments both in normal and splenectomized individuals gave ample demonstration that the spherocyte had a normal life span in a splenectomized individual,[36] but was removed rapidly from the circulation in the presence of an intact spleen.[37] It was found also that passage through the spleen further increased the susceptibility of these spherocytes to lysis by hypotonic solutions.[38] All studies pointed to the fact that removal of the spleen removed the spherocyte-trapping mechanism, thus allowing the erythrocytes of hereditary spherocytosis to have a normal life span.

Hereditary leptocytosis (thalassemia and related syndromes). By hypotonic fragility measurements it was found that the red cells were more resistant than the normal erythrocytes, thus pointing to the lack of value of this test in assessing *in vivo* mechanisms of hemolysis. Survival time studies and autosurvival using Cr^{51}-labeled red cells indicated a variable degree of shortening of life span.[39, 40] In some severe cases (Cooley's anemia), the spleen, if considerably enlarged, was found to increase the hemolytic tendency. Splenectomy under such circumstances often reduced the transfusion requirement considerably without, of course, altering the fundamental nature of the disease.[41] With the use of Fe^{59} and other methods, an ineffective type of erythropoiesis also has been demonstrated in the disease.[42] Thus, from these relatively limited observations, it appears that the anemia of thalassemia is due only partly to increased hemolysis of mature erythrocytes. The findings of heme diversion,[43] that is, the breakdown of primitive erythroblastic cells in the bone marrow, and of ineffective erythropoiesis are probably of as much importance as increased hemolysis.

Sickle cell disease. The increased viscosity of sickle cells under anoxemic conditions and their liability to mechanical trauma within the circulation have been pointed up in several experiments.[44] The tendency to multiple thrombotic manifestations due to the piling up of masses of sickle cells in certain organs is undoubtedly an important feature of the so-called crisis in this disease.[45] Increased hemolysis within an enlarged spleen may play a role in some cases.[46]

significance for the *in vivo* hemolytic state is open to some question, inasmuch

as the very minor changes in pH that take place during sleep are not likely to result in the extreme hyperhemolysis that takes place within the blood vessels All studies point to an intrinsic defect of the red cell, the nature of which is obscure Whether this is due to faulty production or to the activities of a hitherto undiscovered immunological mechanism is by no means clear [49] It is apparent that the spleen plays no role in the hyperhemolysis The considerable work that has been done on various plasma factors, including properdin,[50, 51] indicates that these are normal constituents of the blood plasma acting against an abnormal red cell. Perhaps too much attention has been devoted to the plasma factors and too little to the intrinsic red cell abnormality of the disease

To summarize, the various physiopathological studies of the intrinsic red cell abnormalities, including the more recent studies with radioactive isotopes, have demonstrated quite elegantly many, if not most, of the hemolytic mechanisms involved in the increased hemolysis associated with these conditions However, they have failed to show the "why" of the intrinsic abnormality and thus have failed to approach the fundamental basis for the intrinsic red cell disorder, this is the province of the chemical era we are in now

Chemical Aspects

Hereditary spherocytosis The increased hemolysis of hereditary spherocytes *in vitro* may be reduced by the addition of glucose to the incubation mixture [52] This results in a reduction in the loss of potassium from the cell and a decrease in osmotic fragility Moreover, P^{32} is incorporated in smaller amounts in the hereditary spherocyte than in the normal red cell; this has been shown to be due chiefly to a diminished incorporation into ATP and 2,3-diphosphoglyceric acid [53] It is evident that high energy phosphate compounds, probably necessary for the maintenance of the biconcavic shape of the red cells, are deficient in hereditary spherocytosis, perhaps as the result of a genetically determined enzymatic defect Thus, the hereditary

tion as is the congenital spherocyte However, the mechanical trapping function of the spleen for spherocytes is not to be ignored.

Thalassemia Studies of the red cells in this condition indicate that an [illegible]

Eighteen years later, Pauling and his group[59] originated the concept of the "molecular disease," a disease caused by genetically determined abnormalities of protein synthesis. During the past *nine* years, due to the impetus of Pauling's concept and to the widespread introduction of analysis of hemoglobin by paper electrophoresis, progress in this field has been truly enormous. Several recent reviews give excellent summaries of the clinical and biochemical aspects of these disorders.[60-62]

Some puzzling features of the hemoglobinopathies appear to be near solution. Evidence has accrued to show that the sickle cell trait is due to a mutation by a single gene.[63] Although the various abnormal hemoglobins

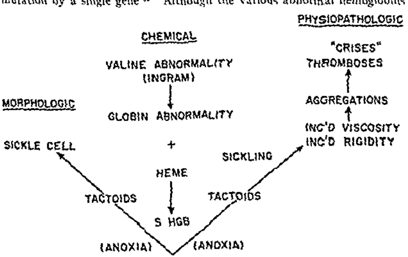


FIGURE 3. Chemical lesion is central: for example, sickle cell disease.

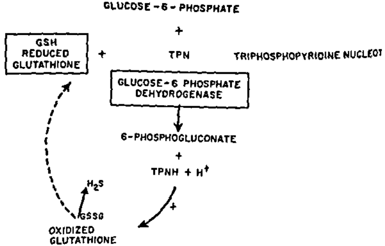
have distinct electrophoretic mobilities, no chemical differences among them were found until recently. Thus, the number of —SH groups of hemoglobins A and S have been found to be identical,[64] as well as the C-terminal amino acid groups,[65] the N-terminal amino acid groups,[66] and the amino acid composition.[67] However, by a unique combination of paper electrophoresis and paper chromatography of trypsin-split fragments of the hemoglobin molecule I[illegible] has demonstrated that hemoglobins S and C differ from A by a [illegible] sequence in one small part of the polypeptide [illegible] are that the amino acid [illegible] of this pattern [illegible] and effects.

Paroxysmal nocturnal hemoglobinuria. Recent studies, [illegible] confirmed, have demonstrated a lipid abnormality in the red cell membrane.[69] Whether this is the primary disturbance or one secondary to some extrinsic phenomenon is obscure. The chemical abnormality, which leads to the morphologic, in turn results in physiopathological disturbances (TABLE 3).

TABLE 3
RED BLOOD CELL ABNORMALITIES

Chemical		Morphologic	Physiopathological
Poorly functioning glycolytic mechanism Poorly functioning ATP cycle	→	Spherocytosis →	Trapping in spleen Short life span
Lack of iron utilization Faulty heme synthesis	Insufficient hemoglobin for cytoplasm →	Target cell (Leptocyte) →	Fragmentation Ineffective erythropoesis Precursor cell hemolysis
Valine abnormality Globin abnormality Abnormal hemoglobin Tactoids	→	Sickle cells →	Rigid Agglutinated (?) Viscous masses

Occult intrinsic defects (FIGURE 4) Our knowledge of hemolytic mech nisms has advanced greatly in the last few years by the finding that cert individuals have chemical defects of the red cells which are brought out o



(1) G-6-P DEHYDROGENASE IS DEFICIENT, PROBABLY ON GENETIC BASIS
(2) GSSG CANNOT BE REDUCED, HENCE RED CELL GSH IS LOW.
(3) INTEGRITY OF RBC MEMBRANE DEPENDENT UPON ADEQUATE AMOUNT GSH THEREFORE CELLS ARE SUBJECTED TO PREMATURE DESTRUCTION.

FIGURE 4 Proposed defect in aniline and fava sensitivity

when the red blood cells are challenged by various chemicals The track down of this occult defect represents a very exciting chapter of mod hematology.

acute hemolytic anemia A peculiar feature of the association between ingestion of such organic chemicals and the development of hemolytic anemia was its almost exclusive occurrence in Negroes [72, 73] Beutler and his colleagues hav[illegible]
They founc
self-limited
red cells.
the red blo
bers of Heinz bodies appear in the blood just prior to the acute hemolytic episode, these appeared both *in vitro* and *in vivo*, and their numbers were increased in the presence of increased amounts of oxygen [77] It was apparent that only the older members of the red blood cell population were affected by primaquine [78] These primaquine-sensitive cells were unusually sensitive to hemolysis by acetanilid, sulfanilamide, thiazolsulfone, phenylhydrazine, sulfoxone, and phenacitin [79] An important finding was that the red cells of these patients were uniformly deficient in reduced glutathione[80] and that a further [illegible]

[illegible] of sensitive cells with the lytic drug [illegible] However, it appears that the deficiency of reduced glutathione is secondary to a deficiency of glucose-6-phosphate dehydrogenase in these red cells [83] This enzyme maintains the substrates necessary for the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) in the presence of glutathione reductase [84] This deficiency of glucose-6-phosphate dehydrogenase may be genetically determined [85]

More recently, the very interesting disorder called favism has been brought into the picture [86, 87] Limited largely to Mediterranean countries and peoples, this mysterious hemolytic disease has been shown to reside in a sensitivity of the red blood cells of certain individuals to various products of the fava bean or the pollinating fava plant. In Israel, Sheba and his collaborators have for some time been impressed with the relative infrequency of this and other hemolytic disturbances in the Ashkenazy Jews and with their relatively high frequency in the Sephardic (Oriental) Jews [88] Their recent studies indicate a hereditary defect of the red blood cells of certain Oriental Jews identical with the defect found in Negroes developing primaquine her[illegible]
Th
thc
life
unusual chemicals that were unknown until the days of modern medicine, or perhaps the fava bean and its products

Extrinsic Defects of the Red Cells as a Cause for Hyperhemolysis

The "Sick Circulation" (Hemopathic Hemolysis)

There are many other factors (chemical, parasitic, bacterial), extrinsic to the red cell, which may destroy it Of them, hemopathic hemolysis (sick

circulation) is of considerable interest. In recent years, many studies using the readily available Cr^{51} technique, have shown that in such illnesses as cirrhosis, nephrosi[illegible]
span develops[89]
toxic factors of [illegible]
destroyed more rapidly than in the normal milieu This gives some illustration of the marked sensitivity of the circulating red cell to relatively minor changes in its environment.

Immunohemolytic anemias The disposal of worn-out erythrocytes by the body represents an interesting problem, since the reticuloendothelial system which is responsible for the removal of such cells, is also at least one source of antibodies Under normal conditions, these functions of the reticuloendothelial system do not overlap, and destruction of the aged red cells does not incite antibody formation The solution of the problem, as envisioned by Burnet, is ingenious, and it is evident that the "immunoclastic system" can discriminate, within very narrow limits, autologous ("self") materials from foreign ("not self") substances The concept of "self" and "not self" implies that "in order to allow . . differentiation [of worn out body cells from foreign organic material] expendable body cells carry 'self-marker' components which allow recognition of their 'self' character Antigens in general are substances of the same chemical nature as the marker components but of different molecular configuration "[90] In other words, the body's own cells are branded in a highly distinctive manner, this labeling scheme is in turn complemented by a censor, the recognition system If the recognition factor corresponds to "self," no reaction occurs other than the normal physiological disposition of worn-out cells, if there is a mismatch between the marker components and the recognition system, however, an immune response is provoked Although highly speculative, these concepts, originally put forth by Burnet and Fenner, are not without some foundation in fact Indeed, it was possible for Burnet, reasoning within the framework of his theory, to predict the phenomenon of acquired tolerance,[91] one of the major discoveries

[illegible]

or (2) an alteration in the self-marker component The former group might include those disorders directly affecting the reticuloendothelial system, such as leukosarcoma or chronic lymphocytic leukemia It is not unusual to find autoimmune hemolytic anemia occurring in these diseases and one might speculate that, when the neoplastic population of cells arises and replaces the normal group of cells comprising the reticuloendothelial system, these new cells, which may have arisen by a series of somatic mutations, possess a recognition system different from the normal cells The dying red cell,

[illegible]

erty *et al.*

The second possible mechanism, namely, an alteration in the self-marker component of the cell might be induced by very mild treatment (FIGURE 5) Enzymes that ordinarily do not come into contact with red blood cells may so alter them if they leave their accustomed channels, either by intent or by accident A number of observations lend support to this speculation the occurrence in serum of autoagglutinins and autohemolysins in patients with *posttraumatic meningeal bleeding,*[93] *hemothorax, or hemoperitoneum,*[94] *the* appearance of a positive Coombs' test in rabbits given intraperitoneal injections of their own blood[95] or by intramuscular injections of such blood mixed with Freund's adjuvant,[96] and the occurrence of autohemolysins in dogs following the intravenous injection of autologous, lysed red blood cells [97]

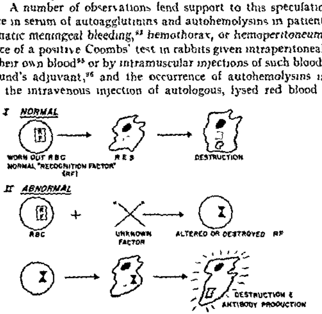


FIGURE 5 Genesis of autoimmune antibodies a working hypothesis

Furthermore, treatment of red blood cells with certain drugs,[98-101] *lipid* solvents,[102] or lead[103] will induce hemolytic anemia with positive Coombs' test

The Nature of the Antibodies in Immunohematological Disorders

Although Moreschi[104] in 1908 reported that, if antiserum against rabbit red cells made in goats was diluted sufficiently so that no agglutination of *the rabbit red cells occurred, very strong agglutination occurred if a drop of* goat serum against rabbit serum was added to the mixture, little attention was paid to this important discovery Thirty-six years later, Coombs and his group described a test which they thought " promises to be of practical importance "[105] In this test, red cells coated by nonagglutinating antibody are rapidly clumped by the addition of a drop of antihuman serum (globulin) prepared in rabbits The introduction and exploitation of this *simple test has revolutionized the understanding and management of iso-* and autoimmune hemolytic anemias [106-109]

It is now known that the material in Coombs' serum responsible for its uncovering effect is a γ-globulin,[110] furthermore, the substance coating the red

cell in most instances of autoimmune hemolytic anemia is a γ-glob
This can be demonstrated in two general ways: (1) by the use of the
globulin reaction[111] in which the action of Coombs serum is nullifie
preincubation with extremely minute amounts of human γ-globulin, a
tion reminiscent of other highly specific immun
tion of the material eluted from the surface of
method, γ-globulins have been found to coat
certain circumstances which will be discussed la

Hemolytic Mechanisms in Autoimmune Hemolytic Anemia (TABLE 4)

The role of the coating antibody How do these varieties of antibody attack the red cells and produce abnormal hemolysis? When blood from these patients is transfused into normal persons, most of the donor red cells have a normal survival,[139] but when normal blood is transfused into a patient with autoimmune hemolytic anemia, the survival of the transfused cells is very short and the donor cells disappear in an exponential fashion,[140] indicating that cell age is not the primary reason for death, but that an extracellular factor is responsible Furthermore, normal cells given to such a patient become coated with antibody prior to their destruction [141] It is important to note that when blood from a patient with autoimmune hemolytic anemia is given to a normal individual, not only do these cells remain sensitized during their life in the normal circulation, but some of the normal recipient's cells also become sensitized It is clear, then, that sensitization per se is not the complete stimulus for increased hemolysis However, it is known that red cell antibody does produce decrease in tensile strength or increase in mechanical fragility of the red cell membrane [142] The accelerated hemolysis of sensitized red cells that occurs *in vitro* is due to progressive degenerative changes in the cell membranes,[143, 144] so that it is possible that the primary action of sensitizing antibodies in these patients is to injure the membrane in an as yet unknown fashion, thereby rendering the cell susceptible to destruction by other mechanisms, normal or abnormal

A striking finding in many cases of autoimmune hemolytic anemia is erythrophagocytosis, which may be seen in the peripheral blood, or in tissue sections [145] The process has been studied by means of tissue culture, injury induced not only by specific immune serum, but also by trypsin will induce erythrophagocytosis A thermolabile serum factor has been found to partake in the process Monkeys suffering from malaria associated with increased hemolysis have been shown to have in their circulation protein-coated red cells, and these coated cells are observed to be instantaneously ingested by phagocytic cells of the liver, while normal, uncoated cells are not phagocyted [146] Whether *in vitro* agglutination of red cells, which can be quite striking in cases of this sort,[147, 148] as well as in the experimental animal,[149] actually represents a hemolytic mechanism remains to be proved

Splenic mechanisms It has been suspected for many years that erythrocyte injury induced by antibody cannot be the entire hemolytic mechanism in autoimmune hemolytic anemia Muir and McNee, almost fifty years ago, clearly recognized that the *in vivo* destruction of red cells by antierythrocytic serum differed qualitatively and quantitatively from the *in vitro* destruction of the cells by the same serum [150] This observation has been confirmed more recently,[151] and it appears that, although the amount of blood destruction is proportionally greater *in vivo* than *in vitro*, hemolysis by the intact animal is more gradual than in the test tube An important feature of experimental immunohemolytic anemias is that blood destruction is diminished in splenectomized animals as compared to normal animals [152] However, the exact role of the spleen is difficult to evaluate in the light of clinical experience and

conflicting experimental data However, there is no doubt, that in man spleen plays a major role in the rapid sequestration of antigen-coated red c as demonstrated by measurement of radioactivity over the spleen after injection of Cr^{51}-labeled red cells [152, 153] When red cells are coated by agglutinating antiserum, the normal spleen rapidly traps almost all of administered cells, about one half of them being sequestered within 30 mi Following their sequestration in the spleen, these sensitized cells are he

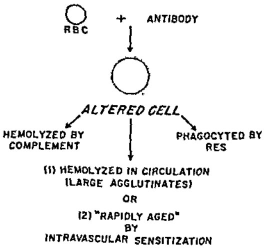


FIGURE 6

the spleens of patients with autoimmune hemolytic anemia [156] It has suggested that the spleen in autoimmune hemolytic anemia contains a stance that sensitizes red cells [157] Although these experiments are impres they do not explain the entire picture, (1) since it is well known that pos

responsible either for actual destruction of red cells or their sensitizatio

former agent although occasionally striking, have been inconstant The remarkable effects of ACTH and the corticosteroids in the great majority of cases of autoimmune hemolytic anemia are probably due to a direct action of these substances on antibody-producing cells The fact that splenectomy is almost uniformly a cure in hereditary spherocytosis, while its removal in autoimmune hemolytic anemia is often a failure may be related to the specialized architecture of the spleen and the effect of this structure on the abnormally shaped red cells of hereditary spherocytosis

Immunohemolytic anemias may thus be conceived of as the end result of the attachment of immune globulin to the red cell surface (FIGURE 6) If the antibody is of the complete or direct-reacting type, as in the A-B-O system, then further reaction with complement results in hemolysis.[164] That this is not a simple system has recently been demonstrated, and the recent evidence indicates a reaction along these lines

$$E + A \rightarrow EA$$

$$EA + C_{1,4} \xrightarrow{Ca} EAC_{1,4}$$

$$EAC_{1,4} + C_2 \xrightarrow{Mg} EAC_{1,2,4}$$

$$EAC_{1,2,4} + C_3 \rightarrow E^*$$

$$E^* \rightarrow \text{ghost} + \text{hemoglobin}$$

In this scheme, E represents the erythrocyte, A the antibody and C_1, C_2, C_3, and C_4 the various components of complement [165]

If the coating antibody is of the incomplete or indirect type, other complex mechanisms are brought into play Evidence for these mechanisms is somewhat circumstantial, but the following changes are probably effected the red cell membrane becomes brittle, intravascular agglutination may be pronounced, thereby inducing further mechanical stresses on the membrane, and the protein-coated cells are rapidly phagocyted by both free and fixed reticuloendothelial cells An unknown factor may cause normal tissue lysins to increase in activity Furthermore, severe crippling chemical changes in the membrane may be induced by attachment of antibody to the cell Actual lysis eventually takes place either within the circulation (intravascular type) or within the cells of the reticuloendothelial system (extravascular or intracellular type), as shown in TABLE 4

TABLE 4

AUTOANTIBODIES—MECHANISMS INDUCING HEMOLYSIS

(1) Tight attachment to RBC surface coating
(2) Chemical, enzymatic, physical changes due to
    (a) Coating
    (b) Its degree
(3) Extrinsic factors aiding hemolysis of coated RBC
    Splenic and other intravascular sequestration
    Trauma of circulation
    Trapping of spherocytes by spleen
    Phagocytosis

To summarize and categorize what has been said above, one may divid immunologic mechanisms into hetero-, iso-, and autoimmune type Heteroimmunity is readily reproduced in the experimental animal In th case of the red cell, various features very similar to those seen in huma immunohemolytic
fragility, polychrom
population, can be
Isoimmunization is seen very commonly in the hemolytic transfusion rea tions and in hemolytic disease of the newborn. Autoimmunization remai a puzzling phenomenon, particularly since as yet it has not been clear demonstrated by experimental means. Although the antibodies that a found in autoimmune hemolytic anemia have all the criteria of immu bodies, the lack of a well-defined antigen is cited in criticism of the immun logical nature of the process That the substance demonstrated on th surface of the red cells and free in the serum is a factor acting *against* norm red cells cannot be denied, likewise, the fact that it acts only against r blood cells This is certainly an *antibody* However, whether it therefo fulfills the full criteria of an immunologist who must know the antigen befo he certifies that an antisubstance is an antibody is another matter, whi inevitably brings one into the realm of semantics. We prefer to call t substance adherent to the red cell and giving a positive Coombs' test a often found free in the serum an antibody.

Although the antibody is not the only factor in hemolysis, it certain appears to be the prime factor It attaches itself firmly to or coats the su face of the red cell This is by no means a harmless procedure, since it m result in agglutination and in a physical and chemical dysfunction of t surface of the red cell Physical modifications have been demonstrated electron micrography [166] Tishkoff has demonstrated a very interesti modification of the δ-aminolevulinic acid concentration following antibo sensitization of the red cell surface [167]

Morphologically, the red cell acted upon by immune body shows sphe cytosis, physiopathologically, it has a shortened life span, in part perha because it is a spherocyte, chemically, various enzymatic and metabo constituents have probably been modified The immune spherocyte pro

cytes, reticular plasmocytes, and, perhaps, combinations of these Or

removed Nitrogen mustard and the alkylating agents used in the attempt to destroy antibody-producing tissue are occasionally effective, but far more constant effects are obtained by use of ACTH and the corticosteroids If these are given in sufficient, usually large, amounts, the concentration of antibody generally is reduced greatly At first, there is disappearance of free antibody from the serum, followed by an actual reduction in the surface of the red cell This actually has been measured,[169] FIGURE 7 Eventually, the Coombs' test may become negative, although this is unusual, in most cases, with continued use of small doses of corticosteroid, the antiglobulin determination remains positive, although the indications of increased hemolysis are lacking This may represent an immunological landmark or may indicate the presence of very small quantities of antibody globulin on the

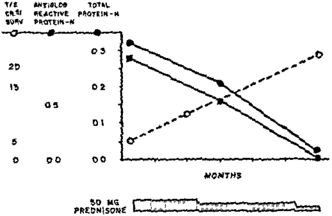


FIGURE 7 Case 1 'idiopathic' autoimmune hemolytic anemia prednisone therapy

surface of red cell, insufficient to cause trouble, other factors may be required for the hemolytic state to recur This is often potentiated by a viral infection or the injection of one of the numerous vaccines now used against viral infections such as poliomyelitis and Asian influenza [164]

Summary

The emphasis in previous years has centered on factors extrinsic to the red cell resulting ultimately in its destruction The wear and tear of the circulation, erythrostasis in the spleen, the phagocytic activity of the reticulo-endothelial system, various lysins, not only those normally indigenous to the circulation but others arising by abnormal mechanisms these were emphasized The red cell was a nonnucleated, amorphous, and highly peculiar mass of cytoplasm that could be destroyed in a passive way With increasing knowledge of the complex chemical and enzymatic structure of the red cell and its many metabolic activities, emphasis has shifted to the cell milieu and thus to a more basic approach

Why certain red cells are intrinsically defective and what happens to red cells when they are attacked by extrinsic mechanisms have become matters of at least partial knowledge. It is now evident that enzyme systems either run down naturally or are attacked, chemical factors become depleted or modified and, ionic transfers are impaired; all of these factors lead to early red cell destruction. Usually, the hypothetical recognition factor present in all red cells keeps it out of trouble, should it become altered, antibodies develop and these may attack their own cells. Thus, hemolytic phenomena are in a dynamic state with many intrinsic phenomena in a state of precarious balance with a host of extrinsic factors How the red cell finally comes to dissolution is not clear, that it stands up so well most of the time is indeed remarkable Perhaps as our chemical and physicochemical knowledge increases, it will be possible to produce modifications of the red cell ultrastructure and thus increase its life span. This should be of considerable help not only in hemolytic disease, but in various other types of anemia in which a productive defect is present

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METHODS FOR THE STUDY OF HEMOLYTIC DISEASE

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I propose to discuss briefly some of the problems encountered in the study : hemolytic disease The discussion will be limited to the methods my ssociates and I use at present and the problems we encounter in our attempts) interpret the data that these methods yield

As a preliminary, I shall review the nature of the system with which we :e dealing The tissue principally concerned is the red cell mass In the ormal state and in most hemolytic states the size of the red cell mass does ot vary During hemolytic crises it may grow smaller and, during periods f recovery, it expands, otherwise, with small fluctuations, it remains constant ı size However, the substance of the red cell mass, the individual red cells, changing continually, with new cells replacing the old, but the size of the ıass remains constant because production and destruction of the cells are alanced exactly It is a system of dynamic equilibrium, and on either side . is complemented by other systems in a similar state of equilibrium The ıass of erythroid tissue in the marrow, the mass of plasma iron that shuttles 'om the phagocytes to the erythroblasts, the mass of bilirubin between the hagocytes and the liver, all of these and others are in equilibrium with the irculating red cell mass Each of them is subject to its own system of con- :ol When the normal state is modified by the presence of hemolytic disease, ıe systems of control react and the changes characteristic of the hemolytic yndrome may appear At the present time we study these changes at three :vels of complexity First is the concentration of the substance in question, or example, we measure hematocrit when we are dealing with the circulating ed cells The second order of methodological complexity involves measure- ıent of the size of the substance in question for example, the volume of irculating red cells The third order of complexity involves measurement f the turnover rate for example, the life span of the circulating red cells 'here is a fourth order that we are only beginning to probe the mechanisms vhereby these systems maintain themselves in dynamic equilibrium

Each of the systems, such as the plasma iron, the bilirubin, and the red ell mass, can be expressed by a simple formula

$$M = IT$$

M is mass, whether it be red cells, reticulocytes, plasma bilirubin, or iron is input, the amount of the substance that enters the system per unit of ime T is the average length of time each particle of the substance remains n the system

With this formula in mind let us review some of the false criteria for the presence of hemolytic disease "The absence of anemia indicated that there ... -bnormal hemolysis" Here the reference is to M, the red cell mass

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the blood do differ from one another, some are old and some are young If the old cells took up more isotope than the young, the normal process of red cell destruction would produce a curve of isotope decay resembling the curve attributed to Cr^{51} elution

When the life span of red cells is studied by observing the elimination of a tag, whether it be an isotope or an antigen, it is not certain whether the cells are lost by hemolysis or by hemorrhage, thus chronic gastrointestinal bleeding may simulate a moderate hemolytic disease The use of Cr^{51} provides a convenient safeguard against this error Chromium is not absorbed from the

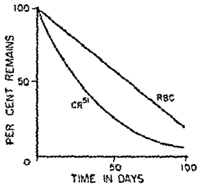


FIGURE 1 Survival of transfused normal red cells as measured by the Ashby method (RBC) and the same cells tagged with radioactive chromium (Cr^{51}) The isotope decays more rapidly than the cells that carry it, this is believed to be due to elution of the chromium

intestine and any Cr^{51} of tagged red cells lost in the intestine ends up in the feces The survival curve of the transfused red cells may be corrected by subtracting the radioactivity of the feces

The loss of red cells by extravasation into other parts of the body is another problem These cells disappear and, if they are tagged, the tag goes with them Should they be considered lost or sequestered? I have encountered situations in the war-wounded where the loss by extravasation (or sequestration) involved a considerable amount of red cells, as much as 1500 ml I have concluded that red cells lost into muscles and tissue spaces are permanently lost and eventually are hemolyzed *Thus any considerable extravasation not only resembles hemolytic disease, but is also one form of hemolytic disease*

The mechanism of hemolysis of extravasated red cells is an interesting problem and, inevitably, it brings up the question of tissue hemolysins The following story bears upon this point

Several years ago I was witness to a conversation between two men who were working on problems of hemolysis The first said that in his laboratory *several years of work had been required to isolate a tissue hemolysin that* proved to be a long-chain unsaturated fatty acid The second man put his tongue in his cheek and said, "We, too, have just isolated a tissue hemolysin

was not diminished This alone does not eliminate the possibility of hemolytic disease By definition, hemolytic disease is a shortened life span within the circulation of the red cells. In other words, the T of the formula is reduced. However, M may remain normal if I is increased to compensate for T, that is, the bone marrow produces more red cells This happens in most hemolytic states When the average life span of the red cells is reduced, there is a compensatory increase of red cell production to prevent anemia If the bone marrow is capable of a full response, a chronic hemolytic process does not cause anemia unless the requirement for new red cells exceeds seven times the normal

Another sign of hemolytic disease is jaundice, but here, again, there is room for misinterpretation of the data. "The absence of bilirubinemia indicated that the anemia was not due to hemolysis" It is true that an increased rate of red cell destruction is associated with an increased rate of bilirubin production, however, it does not follow necessarily that the concentration of plasma bilirubin need be elevated. With reference again to the formula $M = IT$, input (or bilirubin production) may be increased, but it will not cause an increase of M (plasma bilirubin) if T (the time that bilirubin remains in the plasma) is diminished correspondingly. One must be especially careful in drawing conclusions from experiments in animals in which the normal plasma bilirubin is nearly zero.

Reticulocytosis is another sign of hemolytic disease, but the absence [illegible] reticulocytes does not imply necessarily an absence of hemolytic disease. Even at high rates of production, only a few red cells appear as reticulocytes when the bone marrow is suffering from a lack of iron or vitamin B_{12} [illegible] well-compensated hemolytic disease, when the bone marrow has increased its output sufficiently to prevent anemia, red cell production may be found to be five times the normal rate, but the reticulocyte count is within the normal range, or slightly increased.

These examples of anemia, bilirubinemia, and reticulocytosis demonstra[illegible] some of the signs that cannot be relied upon to reveal a hemolytic process [illegible] only moderate intensity. In each instance they involve a measurement [illegible] concentration, but little more would be gained by measuring the total volum[illegible] To detect the mild or moderate hemolytic process, a measurement of turnov[illegible] rate is often needed

It is possible to study the turnover rate of red cells themselves or of su[illegible] constituents as iron and porphyrin. The data, even from such eleg[illegible] experiments, must be regarded with suspicion and interpreted with ca[illegible] For example, consider the measurement of red cell life span by means [illegible] autologous cells tagged with Cr^{51}. It is known that the isotope is lost m[illegible] rapidly than are the cells that carry it (FIGURE 1) When this phenomen[illegible] was first demonstrated, it was ascribed to elution. The red cells w[illegible] believed to shed a little of their chromium each day. However, if there w[illegible] different kinds of red cells in the blood and some of them accepted m[illegible] chromium than others during the tagging procedure and if the transfu[illegible] cells with much Cr^{51} were destroyed late, then the curve of isotope de[illegible] would resemble the one attributed to elution of the isotope The cells

the blood do differ from one another, some are old and some are young. If the old cells took up more isotope than the young, the normal process of red cell destruction would produce a curve of isotope decay resembling the curve attributed to Cr^{51} elution.

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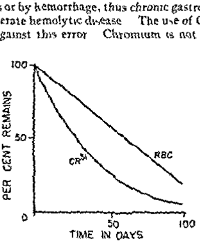


FIGURE 1. Survival of transfused normal red cells as measured by the Ashby method (RBC) and the same cells tagged with radioactive chromium (Cr^{51}). The isotope decays more rapidly than the cells that carry it, this is believed to be due to elution of the chromium.

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We minced the tissue and put it into a distilling apparatus. When we heated tissue to 100° C a very powerful hemolysin came through the condenser" "Oh," said the first man, "but that was nothing but distilled water" "True enough, and your fatty acid is nothing but soap. Why not take your soap and my water and wash our hands of tissue hemolysins?"

This was not empty sarcasm Water, as it exists in tissues, is not distilled water and it is not hemolytic Fatty acids, as they exist in tissues, are not soaps and they are not hemolytic The best evidence bearing on the fate of red cells in tissues is the direct microscopic observation of extravasated red cells *in vivo* E. L. Clark has repeatedly observed that red cells lost in the extravascular tissue spaces are not lysed, but may remain intact for days or weeks The "hemolytic factor" that finally destroys them is the wandering phagocyte, the so-called tissue macrophage or blood monocyte

It is gratifying to note that the investigators of hemolytic disease are beginning to pay more attention to the role of phagocytes In the past we have lived too much in the test tube, seeking hemolysins and agglutinins to explain the pathogenesis of red cell destruction In some diseases these are important, and where they are important we find the direct evidence of their activity an elevated plasma hemoglobin The direct destruction of red cells by antibody activity is an intravascular event, and hemoglobin of the cell is spilled into the plasma In most forms of hemolytic disease much, if not all, hemolysis occurs otherwise The plasma hemoglobin is not remarkably high because the abnormal hemolysis is accomplished by the phagocyte. When radioactive red cells are involved, the radioactivity accumulates in the phagocytic organs, the spleen, and liver Such hemolytic mechanisms are not easily susceptible to study in the test tube. The readily available phagocytes, the polys and monocytes of the blood, are not implicated. The

greatly refined by the use of isotopes It is possible to study the rate at which the constituents are transported (and presumably utilized), and it is also possible to study the rate at which the constituents are incorporated into the red cell mass.

porate the glycine into the porphyrin rings of the hemoglobin molecules. single large dose of N^{15} glycine is given Thereafter, for about a week, the concentration of N^{15} in the porphyrin of the red cell mass increases as new red cells are delivered from the bone marrow When all the glycine has been utilized the concentration of isotope in the red cells ceases to grow and, in the normal state, it begins to decline after about 80 or 90 days. Gradually it disappears and a mathematical treatment of the data demonstrates that the life span of the red cells is about 120 days This agrees well with information obtained by the Ashby method. Suppose the same dose of N^{15} glycine

is given to a patient with hereditary hemolytic disease. The rate of incorporation is more rapid. The maximum level achieved by the circulating red cells is greater and the decline occurs rapidly. Mathematical treatment of these data indicates a *life span* of the red cells to be about 40 days, a figure that is 3 times greater than that obtained by the Ashby method. Why the discrepancy? The *time required* for incorporation of the isotope is important. During the week when the concentration of isotope achieves its maximum level in the red cells, many red cells are formed and destroyed. The

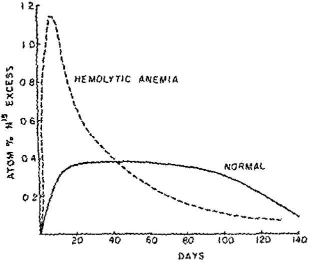


FIGURE 2. Incorporation of glycine N^{15} into the *heme* of circulating red cells. The normal curve indicates a 120-day cycle. The curve in hereditary hemolytic anemia indicates a 40 day cycle. Actually, the *red cells* in this disease have an average life span of 14 days. This isotope *incorporation method* does not give an accurate *measure* of red cell life span in severe hemolytic disease because it cannot account for the short lived cells that are created and destroyed during the first 10 days while the isotope concentration is building up in the circulating red cells.

average life span of these *red* cells is only 14 days, many of them live much less than that. The *mathematical* treatment of isotope concentration does not take into account those red cells that appeared and were destroyed during the build up of *isotope concentration*.

The turnover rate of plasma iron has been related to the rate of hemoglobin synthesis. The assumption is made that most of the plasma iron is in [illegible]

by giving an intravenous tracer dose of Fe [illegible] and following its disappearance by serial sampling. When the concentration of iron has been measured and the plasma volume is known, the turnover rate can be expressed in terms of

We minced the tissue and put it into a distilling apparatus. When we heat tissue to 100° C. a very powerful hemolysin came through the condenser "Oh," said the first man, "but that was nothing but distilled water" "Tr enough, and your fatty acid is nothing but soap. Why not take your so and my water and wash our hands of tissue hemolysins?"

This was not empty sarcasm. Water, as it exists in tissues, is not distil water and it is not hemolytic Fatty acids, as they exist in tissues, are soaps and they are not hemolytic The best evidence bearing on the f of red cells in tissues is the direct microscopic observation of extravasated cells *in vivo* E. E. Clark has repeatedly observed that red cells lost in extravascular tissue spaces are not lysed, but may remain intact for days weeks The "hemolytic factor" that finally destroys them is the wander phagocyte, the so-called tissue macrophage or blood monocyte.

It is gratifying to note that the investigators of hemolytic disease beginning to pay more attention to the role of phagocytes. In the past have lived too much in the test tube, seeking hemolysins and aggluti to explain the pathogenesis of red cell destruction In some diseases t are important, and where they are important we find the direct evidenc their activity: an elevated [illegible]
cells by antibody activity
cell is spilled into the plasr
not [illegible] not rema
bly
Wh
the phagocytic organs, the spleen, and liver Such hemolytic mechan are not easily susceptible to study in the test tube. The readily avai phagocytes, the polys and monocytes of the blood, are not implicated culprit is the fixed phagocyte that lines the sinusoids of the spleen and l its abnormal appetite in hemolytic diseases is little understood.

The study of turnover rates of red cells and their constituents has greatly refined by the use of isotopes It is possible to study the ra which the constituents are transported (and presumably utilized), and also possible to study the rate at which the constituents are incorporated the red cell mass

While isotope incorporation methods are of value, sometimes they peculiar limitations when applied to the study of hemolytic disease [illegible]

[illegible]
the normal state, it begins to decline after about 80 or 90 days. Grad it disappears and a mathematical treatment of the data demonstrates the life span of the red cells is about 120 days. This agrees well with info tion obtained by the Ashby method Suppose the same dose of N^{15} gl

been found between the turnover of iron and the turnover of circulating hemoglobin

My discussion has concerned itself with the systems related to the red cell mass and hemolytic disease and with methods and measurements made at three levels of complexity concentration, volume, and rate I have stressed the importance of recognizing the limitations of our methods when attempting to interpret results The mechanisms of the fourth level, which involve the mechanisms whereby these dynamic systems are maintained in equilibrium, are almost completely unknown Even the mechanism whereby the red cell mass is held at a constant level is not known Given a population of red cells with normal lifespan, the size of the red cell mass must be controlled by adjusting the rate of production In physiological systems the dynamic steady states, such as body temperature, water balance, or red cell mass, depend upon feedback mechanisms Mechanical models of these mechanisms are commonplace when the room temperature falls, this information is fed back to the furnace through a thermostat and the heat continues until the room is at the proper temperature again When the size of the red cell mass shrinks, this information is fed back to the bone marrow and red cells are released until the mass is normal and the bone marrow is "turned off" All feedback systems depend upon the oscillation of some factor that is capable of producing a stimulation In the system that maintains the size of the red cell mass, it is not known what factor oscillates to cause the stimulation the red cell volume itself, oxygen tension of the blood, oxygen tension of the tissues, hemoglobin concentration of the blood, total blood volume, or the concentration of some end product of red cell destruction The amplitude of oscillation may be so small that it cannot be measured by methods now available

It is evident that many factors are capable of modifying the erythropoietic activity of the bone marrow As the study of this complex area increases, it will be well to keep in mind the concept of *fundamental* control of erythropoiesis and to distinguish this from other phenomena that reflect only a modification of the fundamental system Several examples are

(1) A patient develops a craniopharyngioma that destroys his pituitary His red cell mass dwindles from a normal value of 30 ml /kg to 20 ml , and there it remains The life span of his red cells is unaltered, his marrow is producing one third less hemoglobin, but a dynamic steady state is retained, functioning at a lower level

(2) A patient in good health and having hereditary spherocytosis is found to have a red cell mass of 30 ml /kg and a hematocrit of 48 per cent, the average life span of his red cells is 15 days Therefore, his marrow is producing 8 times the normal quantity of red cells The dynamic steady state is maintained by a very high rate of marrow activity, but the stimuli of anemia and anemic hypoxia are lacking

(3) A miner living in the Andes at 4000 meters has a red cell mass of 45 ml /kg and his red cells have a normal life span Although his tissues continue in a state of moderate hypoxia, his marrow is producing only 1 5 times the normal output, presumably, it is capable of 8 times the normal

milligrams of iron per day. The hemoglobin molecule contains 4 atoms o[illegible] one-third per cent of its bulk. Thus, 1 mg of iron [illegible] formulas it is po[illegible] ure would be accu[illegible]
rate if all of the plasma iron went into hemoglobin and if there were no othe[r] source of iron In the normal state there is good correlation between the figures for hemoglobin synthesis and those derived by other methods How[ever], in some varieties of hemolytic disease there is a wide discrepanc[y]. There is a possibility that iron may be bootlegged in the bone marro[w]. Phagocytic cells may destroy erythrocytes and surrender the iron direct[ly] to adjacent erythroblasts without passing through the plasma channe[l]. This is possible, but the discrepancies lie in the other direction: too much iro[n] [illegible] ...ible to accou[nt]

globin that is formed For example, if hemoglobin or erythroblasts... ing hemoglobin were destroyed in the bone marrow, the "aborted" pigme[nt] would not be measured by methods dealing only with the blood Th[is] probably takes place to a small extent even in the normal situation, becau[se] the normal marrow may produce a few imperfect cells However, while [it] is true in hemolytic disease that cells may be destroyed in the marro[w] there is another and obvious cause for the disproportionate rate of iron tur[n]over This is the siderocyte.

The siderocyte is a red cell that contains one or more granules of hem[o]siderin iron This represents iron taken into the erythroblast but ne[ver] used for hemoglobin In the normal state, only a few siderocytes are del[iv]ered from the marrow to the blood In some varieties of hemolytic anem[ia] two thirds of the red cells may contain iron granules The siderocy[te] receives very particular attention from the spleen. Somehow the spleen [illegible] the red cells that c[ontain] [illegible] ne marrow throu[gh] [illegible] rocyte moves i[n] precise parallel with the hemoglobin iron. It is incorporated into the eryth[ro]cyte, it moves with the erythrocyte into the circulating blood, at the end [of] its life span it is removed from the circulation by the RES, and it is return[ed] to the bone marrow by way of the plasma iron channel. It differs fr[om] hemoglobin iron only in that its life span is much shorter, where the spl[een]

THE NATURE OF ERYTHROPOIETIN

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Following the original investigations of Borsook[1] and Gordon,[2] numerous investigators have confirmed the fact that acidified, boiled plasma filtrates from anemic animals and from man when stricken with certain anemias, are capable of stimulating erythropoiesis From our own studies we feel that the activity of these filtrates is due to a single humoral agent that is necessary for normal red cell production We have accordingly sought to identify this single humoral agent

Our previously published investigations[3-5] revealed the protein nature of the erythropoietic agent, erythropoietin, and demonstrated that it had the physicochemical properties of an acid glycoprotein We have subsequently designed fractionation procedures, utilizing cellulose ion-exchange columns, and have isolated an acidic glycoprotein that, when injected into the experimental animal, induces a physiological erythropoiesis [6]

In testing for activity, we have avoided the use of hypophysectomized, starved, or otherwise specially treated animals for two reasons First, we have felt that such preparations have an altered erythropoiesis that may or may not respond in normal fashion to an erythropoietic agent Second, any agent that is the physiological governor of erythropoiesis should be capable of producing a measurable erythropoietic response in a normal animal

The starting material was erythropoietically active, acidified, boiled plasma filtrate (APF) prepared from phenylhydrazine-anemic rabbits by the method of Gordon [2] Following dialysis and lyophilization, the APF was fractionated on a diethylaminoethyl (DEAE) cellulose ion-exchange column, as summarized in FIGURE 1 Fraction pools as designated in FIGURE 1, unmodified APF starting material, and control buffer solutions were injected into rats for assay of erythropoietic activity The results of the assay are presented in TABLE 1

Erythropoietic activity was confined almost entirely to Fraction *D* The minimal response induced by Fraction *C* is attributed to contamination by a small amount of the active fraction From the character of the effluent diagram it appears that the material in Fraction *D* is homogeneous On paper electrophoresis in Veronal buffer (*p*H, 8 6, ionic strength, 0 075) it migrates as a single component with a mobility between that of alpha-2 and alpha-1 globulin It stains as a glycoprotein and does not stain for fat Judging from its behavior on the DEAE-cellulose column, it has a low iso-electric point, preliminary work suggests that it is probably less than 3 9 That it is of small molecular size is indicated by the failure to sedimen[t] actively when APF is centrifuged at 103,000 g for 24 hours Its protei[n] content determined by the method of Warburg and Christian[7] is 69 3 pe[r] cent Its neuraminic (sialic) acid content as determined by Winzler

In each of these cases the fundamental control of erythropoiesis has been modified, but without revealing what that mechanism may be. Apparently it is not a pituitary hormone and it is independent of anemia or hypoxia. When one uses experimental methods that evoke such modifications of the rate of erythropoiesis, one should be careful when interpreting the results in terms of *fundamental* control of erythropoiesis.

that reported for other serum glycoproteins,[8] and is probably responsible for its low isoelectric point. Fraction *C* contains 9 per cent neuraminic acid, while Fractions *A* and *B* each contain 6 per cent

Calculations based on the amount of the starting material and the yield of Fraction *D* indicate that the active fraction stimulates erythropoiesis when injected in 50-μg quantities Preliminary dose response data suggest that

TABLE 1
ERYTHROPOIETIC ACTIVITY OF POOLED COLUMN FRACTIONS*

Fraction pool	Reticulocytes†‡ (percentages)	Fe^{59} incorporation†‡ in RBC (percentages)
A	2 7 ± 0 6 (4)	28 3 ± 6 6 (4)
B	2 5 ± 0 4 (4)	26 9 ± 4 5 (4)
C	2 6 ± 0 4 (4)	29 6 ± 1 0 (4)
D	6 5 ± 1 3 (4)	47 6 ± 2 5 (4)
APF	3 5 ± 0 5 (4)	40 7 ± 6 9 (4)
NaCl-acetate control	2 3 ± 0 4 (4)	21 0 ± 2 6 (4)

* Groups of white female, Sprague Dawley rats (average weight, 220 gm) were injected subcutaneously daily for 4 days with 0 5 mg (by protein content) of respective fractions On day 4 the animals were injected I V with Fe^{59} and the percentage of the injected dose appearing in the total red cell mass in 24 hours was determined Reticulocyte counts were done on tail blood using the dry cresyl blue method

† Mean ± S D

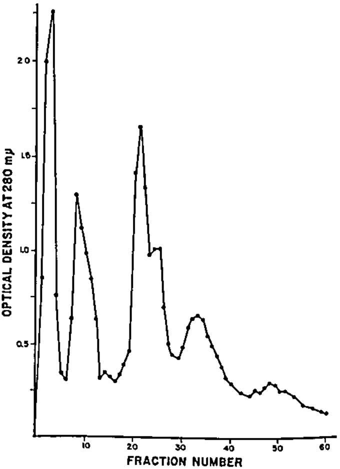
‡ Figures in parentheses indicate the number of animals in a group Underlined values are statistically significant (p value is 0 01 or less)

the injection of 10 μg per day of erythropoietin will induce an erythropoietic response

This characterization of erythropoietin doe[illegible] rrespond to the findings of Linman and Bethell[10] of [illegible] to the concept of Linman[11] that [illegible] [illegible] ty in either the ether [illegible] Thus we have been [illegible] concept of two erythropoietic [illegible] labile, the other relatively heat-stable [illegible], we feel that there is but a single erythropoietin, and that [illegible] a glycoprotein that behaves in the experimental animal as a physiological regulator of erythropoiesis

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modification of the method of Ayala *et al.*[a] is 15.6 per cent. Its hexose co
tent by the anthrone reaction is 7.7 per cent as glucose equivalent, and
glucosamine content by a modification of the method of Elson and Morga
is 10 per cent
suggest that
aminic acid

Part III Hemopoietin

ERYTHROPOIETIC FACTOR IN THE CONTROL OF RED CELL PRODUCTION*

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Oxygen transfer from the atmospheric air to the cytochrome system in the cells is accomplished by the integrated action of a number of organ systems Any impairment in the supply of oxygen to the cells is associated with compensatory changes in the functions of these systems in the pulmonary vital capacity, in the cardiac output, in the production of red blood cells, and in the distribution of blood among the tissues

Undoubtedly the degree of tissue oxygenation must be influential in the mobilization of these compensatory mechanisms Since the principal function of red blood cells is to synthesize, carry, and protect oxygen-carrying hemoglobin, it appears most probable that the degree of tissue oxygenation not only influences but actually controls red cell production

All clinical and experimental observations on red cell production are compatible with this basic hypothesis They have shown that, although the nutritional and hormonal environment conditions the rate of red cell production and hemoglobin synthesis, an anoxic stimulus is necessary in order to balance red cell production with the body's need for oxygen

Mobilization of the Anoxic Stimulus

The compensatory change in erythropoietic function induced by an anoxic stimulus does not facilitate oxygen transfer until days or weeks after it is initiated Consequently, the first line of defense against changes in the tissue oxygen supply is composed of the pulmonary and cardiovascular systems These defenses are in fact so effective that, if properly mobilized, they could restore tissue oxygen supply to normal and eliminate the anoxic stimulus that otherwise would have called the bone marrow into action Such a complete compensation probably takes place in dilution anemia,[1] as suggested by the following experiment

Large amounts of 6 per cent dextran were administered intravenously to normal rabbits over a 4-day period, resulting in (1) a dilution anemia with a decrease in the oxygen-carrying capacity of blood and (2) a hypervolemia inducing an increase in the cardiac output FIGURE 1 charts the mean reticulocyte and bone marrow response in 4 rabbits with dilution anemia and in 5 rabbits in which a comparable anemia was induced by bleeding The lack of an erythropoietic response in dilution anemia suggests that the

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out normal activities, despite anemia, without any increase in cardiac output Concomitantly, there is a strong anoxic stimulus of red cell production It would seem likely that this stimulus does not originate in the protected tissues, but must come from an area not benefited by the redistribution of blood

The location of that area is not known It could be the skin or the kidneys, which are known to be donor areas in the redistribution of blood during anemias or it could be the bone marrow itself

Usually, for the sake of simplicity, the bone marrow has been assumed to respond to anoxia directly with an increase in red cell production This theory is hard to prove or disprove, but direct measurements of the oxygen tension in the bone marrow during anemias have shown the tension to be normal rather than decreased [3] Recently a factor capable of stimulating red cell production has been demonstrated in serum from animals with anemic or anoxic anoxia The existence of such an erythropoietic factor or erythropoietine indicates that the physiological control of red cell production involves at least three elements the erythropoietic factor, an oxygen-sensitive organ that regulates the production or release of this factor, and the erythroid tissue in the bone marrow

The Erythropoietic Factor

The existence of an erythropoietic factor has been suspected for more than fifty years, actually, however, it was not demonstrated until a few years ago The reason for this delay seems to be that serum even from anemic animals contains very small amounts of erythropoietic factor that are hard to measure with ordinary bio-assay techniques Only when massive quantities of serum were bio-assayed was it possible to demonstrate significant erythropoietic activity of serum from animals exposed to anemic or anoxic anoxia As an example, FIGURE 2 compares the reticulocyte response of normal rabbits infused with a total of 200 ml of serum from normal rabbits and from anemic rabbits

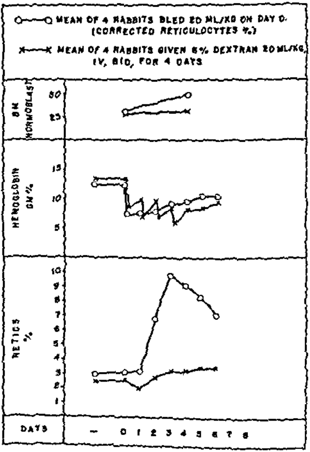
Further progress has been slow, probably because the erythropoietic factor has had to be measured by bio-assay, and no exact method exists to evaluate small changes in red cell production [4]

Although theoretically an excellent measure of [illegible] n,
the reticulocyte count is influen[illegible] y
release of reticulo[illegible] r
rate [illegible]

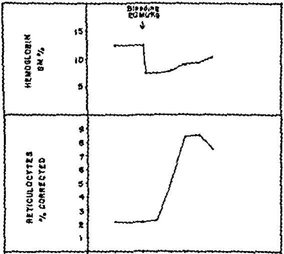
[illegible] radio-
[illegible], in rabbits this test
[illegible]celerated red cell production since
[illegible]porate up to 50 per cent of the iron into red cells

These three methods for measuring red cell production have been used in rabbits bled 20 ml kg and the results are tabulated in FIGURE 3 All three

anoxic stimulus necessary for the induction of an accelerated red cell produ tion can be abolished completely by an increase in cardiac output. Th stimulus is obviously not abolished in ordinary bleeding anemia, and th



such as its heat stability has not been solved to everyone's satisfaction. Some report that the factor is heat-stable, while others report that a considerable amount of its activity is lost after boiling. Utilizing normal rabbits as recipients and the reticulocyte count as a measure of erythropoietic response, the activity of serum from anemic rabbits was found to be reduced



DAYS		~	0	1	2	3	4	5
HEMATOCRIT		41.1		25	26.8	26.5		
SERUM IRON µG %		204		192	154	137		
T/½ MIN		68.8		42	21.9	21.8		
PLASMA IRON TURNOVER MG/DAY/100 ML		1.75		3.49	5.15	4.63		
RED CELL UTILIZATION Fe^{59} %	1 HR	4		18	29	38		
	24 HRS	39		88	94	95		
	48 HRS	65		100	100	100		
	72 HRS	76		100	100	100		

FIGURE 3. Normal rabbits were bled 20 ml/kg with immediate replacement of the lost blood volume by 6 per cent dextran. Changes in blood production were assessed by means of reticulocyte count, plasma iron turnover and red cell utilization and the mean values are recorded.

below a level detectable with this technique after heating to 100° C for 15 to 20 min (FIGURE 4). Obviously, further studies and, most of all, a better bio-assay technique are needed in order to identify and isolate the erythropoietic factor.

Action of Erythropoietic Factor on the Bone Marrow

The anoxic stimulus which, according to our working hypothesis, is mediated by means of an erythropoietic factor, is capable of reactivating yellow

will give a rough estimation of the rate of red cell production and can be used in the bio-assay for erythropoietic factor, but better methods are needed badly

In order to improve the bio-assay technique, recipient animals with a low erythropoietic activity and, consequently, a low twenty-four-hour utilization of iron have been used. To accomplish this, recipient animals have been

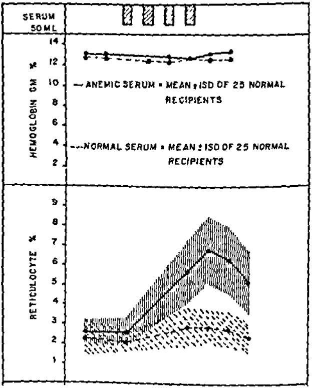


FIGURE 2 The broken lines chart the mean hemoglobin concentration and the mean reticulocyte counts ±1 standard deviation of 25 normal rabbits receiving intravenously 50 ml a day for 4 days of serum from normal rabbits The solid lines chart the mean hemoglobin concentration and the mean reticulocyte counts ±1 standard deviation of 25 normal rabbits receiving intravenously 50 ml a day for 4 days of serum obtained from rabbits with blood-loss anemia

hypophysectomized, starved, irradiated, or hypertransfused Hypertransfusion is the soundest method since it will suppress the endogenously produced erythropoietic factor making the bone marrow more sensitive to the bio-assayed material On the other hand, in hypophysectomized, starved, and irradiated animals the metabolism and reactivity of erythroid tissue have been altered, making it more difficult to evaluate changes induced by the bio-assay of organic materials.

These theoretical and technical problems have interfered with the biochemical identification of the erythropoietic factor Even a simple problem

endered anemic for a period of 20 hours. Following this period 1 group was retransfused in order to terminate the anemic anoxia, 1 group was kept anemic, and 1 group was exposed to 68 per cent oxygen. FIGURE 6 shows that the mean reticulocyte count was the same in the 3 groups on day 2, and only slightly different on day 3. This would mean that the accelerated production of new red cells initiated during the 20-hour period of anemic anoxia

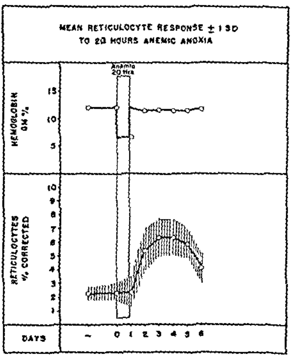


FIGURE 5. Mean hemoglobin [illegible] ion and mean reticulocyte counts ±1 standard deviation of 10 [illegible] on day 0 with dextran replacement. Twenty [illegible] reinfused. The reticulocyte counts on day 1 [illegible]

[illegible] rate for almost 48 hours without being influenced materially by an anoxic, normal, or hyperoxic environment.

These observations and considerations strongly suggest (1) that the division and maturation of the nucleated red cells are processes not conditioned or controlled by the anoxic stimulus, and consequently (2) that the anoxic stimulus operates solely by differentiating multipotential stem cells into pronormoblasts.

Examination of bone marrow during periods of increased and decreased red cell production support these conclusions. Steele[7] found a fixed ratio between pronormoblasts and normoblasts of rabbits before and during chronic

bone marrow Consequently, it appears that the anoxic stimulus can chang multipotential stem cells into nucleated red blood cells In addition to th effect, it has been assumed that the anoxic stimulus accelerates the divisic and maturation of nucleated red cells.

In order to determine the validity of this assumption, normal rabbits we bled 20 ml /kg, kept anemic for 20 hours, and retransfused with the pr viously removed blood FIGURE 5 shows the mean reticulocyte respon

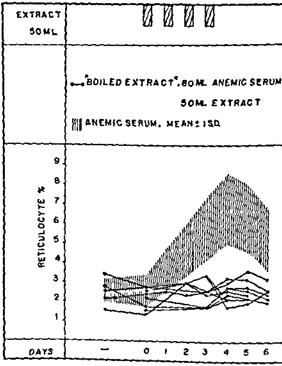


FIGURE 4 Shaded area represents mean reticulocyte counts ±1 standard devia of 25 normal rabbits receiving 50 ml a day for 4 days of serum obtained from anemic bits Lines with solid circles chart individual reticulocyte counts of 8 normal rab receiving 50 ml a day for 4 days of boiled serum extract obtained from anemic rab Eighty ml of serum yielded 50 ml of extract after acidification, boiling for 20 min, centrifugation

induced by the 20-hour period of anemic anoxia The reticulocyte co remained essentially unchanged for the first 24 hours, after which it rapidly, reaching a maximum on days 3 and 4 The rise in the numbe reticulocytes reflects increased mitotic activity of the nucleated red c both during and following the 20-hour period of anemic anoxia To what extent the increased mitotic activity initiated in the period of anemic anoxia is influenced subsequently by the presence or absence of a sustained anoxic stimulus was examined in the following study. Three groups of rabbits were

This hypothesis rules out the endocrine organs as the source of the erythropoietic factor since red cell production continues, although at a reduced rate, after the complete removal of these organs.[10] The spleen, likewise, is not a necessity for red cell production. The reticuloendothelial system, the liver, and the central nervous system have not been studied closely enough to arrive at any definite conclusion as to their erythropoietic significance. However, it has been shown that blockade of the phagocytic properties of

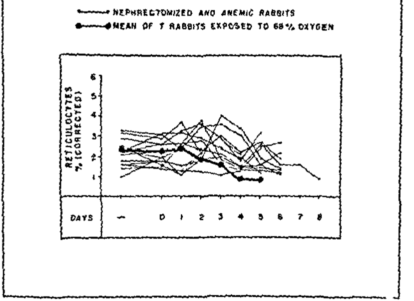


FIGURE 7 Fine lines chart individual reticulocyte counts of [illegible] d rabbits Heavy line charts the mean [illegible] o 68 per cent oxygen [illegible]

[illegible] possibility [illegible] system might play a role [illegible] production

[illegible]ently, Jacobson and his co-workers[11] have suggested that the kidneys control red cell production by releasing erythropoietine in response to anoxia This attractive hypothesis was based on the findings that nephrectomized rats challenged with cobalt or anemic anoxia failed to respond normally with an increase in erythropoietine, whereas ureter-ligated animals responded in the same way as normal animals Furthermore, the hypothesis was supported by the well-known observations that renal disease usually is associated with an aregenerative anemia and that kidney tumors occasionally are associated with polycythemia Unfortunately, similar studies on

bleeding anemia Huff and his co-workers[8] found a similar fixed ratio in people before and after acclimatization to high altitudes Furthermore, studies utilizing colchicine[9] have indicated that when pronormoblasts first

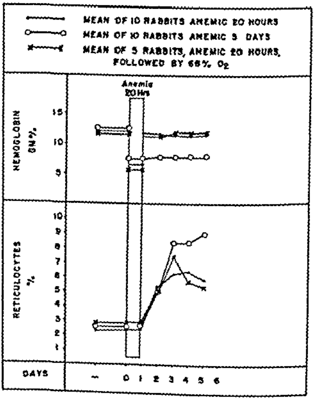


FIGURE 6 Line with solid circles charts the mean hemoglobin and reticulocyte response of 10 normal rabbits bled 20 ml /kg and kept anemic for 20 hours before retransfusion Lines with open circles chart the mean hemoglobin and corrected reticulocyte response of 10 normal rabbits bled 20 ml /kg with dextran replacement and maintained anemic by small bleedings on day 3 and day 4 Lines with crosses chart the mean hemoglobin and reticulocyte response of 5 rabbits bled 20 ml /kg with dextran replacement, retransfused 20 hours later, and then kept in 68 per cent oxygen for the next 4 days

are formed they will divide and mature into red cells at a fixed rate independent of the presence or absence of tissue anoxia

Production of Erythropoietic Factor

It is probable that red cell production is influenced by a number of interacting factors produced or released by a number of tissues or organs However, in order to unravel the intricate process that determines the rate of red cell production, it is useful to start with the hypothesis that one tissue system is responsible for the release of the factor that controls red cell production.

This hypothesis rules out the endocrine organs as the source of the erythropoietic factor since red cell production continues, although at a reduced rate, after the complete removal of these organs [10] The spleen, likewise, is not a necessity for red cell production The reticuloendothelial system, the liver, and the central nervous system have not been studied closely enough to arrive at any definite conclusion as to their erythropoietic significance However, it has been shown that blockade of the phagocytic properties of

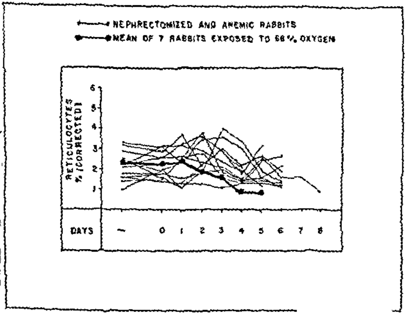


FIGURE 7 Fine lines chart individual [illegible] ed rabbits Heavy line charts th[illegible] o 68 per cent oxygen

[illegible] possibility [illegible] system might play a role

[illegible] and his co-workers [11] have suggested that the kidneys [illegible] red cell production by releasing erythropoietine in response to anoxia This attractive hypothesis was based on the findings that nephrectomized rats challenged with cobalt or anemic anoxia failed to respond normally with an increase in erythropoietine, whereas ureter-ligated animals responded in the same way as normal animals Furthermore, the hypothesis was supported by the well-known observations that renal disease usually is associated with an aregenerative anemia and that kidney tumors occasionally are associated with polycythemia Unfortunately, similar studies on

nephrectomized rats by Mirand and Prentice[12] have not confirmed the experimental findings

In this laboratory all studies thus far have been compatible with the theory that it is the known suppressive effect of uremia on red cell production rather than the lack of kidney tissue that is responsible for impaired erythropoietic function in nephrectomized animals. However, the evidence is indirect since it has been impossible, despite intensive artificial dialysis, to prevent the development of some degree of uremia in nephrectomized animals

In the preuremic phase, nephrectomized rabbits were found to respond to an acute anemic anoxia with the release of erythropoietic factor into serum

TABLE 1

Operation	Bleeding	No	N P N	Hematocrit	Plasma iron turnover mg /day/100 ml	Red cell utilization 24 hr percentage
Sham	0	7	45	39	2 97 ± 0 73	56 ± 10
Bilateral nephrectomy	0	11	94	39	2 11 ± 0 97	55 ± 14
Sham	20 ml/kg	4	47	24	5 24 ± 1 08	72 ± 28
Unilateral nephrectomy	20 ml/kg	4	49	24	5 39 ± 0 69	73 ± 12
Bilateral nephrectomy	20 ml/kg	9	86	20	2 76 ± 0 91	65 ± 13
Bilateral ureter ligation	20 ml/kg	5	92	25	2 81 ± 0 56	81 ± 18

When severe uremia occurred three days after nephrectomy, erythropoietic activity was no longer found in serum, despite severe anemia[11] Furthermore, the reticulocyte counts of nephrectomized rabbits were observed to decrease slowly while the reticulocytes of rabbits exposed to an atmosphere with 68 per cent oxygen decreased at a faster rate (FIGURE 7). If the kidney actually controlled red cell production, nephrectomy should have been followed by cessation of red cell production and disappearance of the reticulocytes much more rapidly than when the red cell production was decreased by breathing 68 per cent oxygen

In order to determine accurately the effect of kidney tissue on red cell production it is necessary to estimate the erythropoietic activity that takes place in the short period after the suppressive effect of surgical nephrectomy has worn off and until the suppressive effect of uremia sets in TABLE 1 summarizes plasma iron turnover and red cell utilization of radioactive iron measured, twenty hours after bilateral nephrectomy, at a point where only moderate azotemia was present These values are compared with values obtained in sham-operated, in unilaterally nephrectomized, and in ureter ligated animals It is apparent from the table that unilateral nephrectomy does not impair the erythropoietic response to an acute hemorrhage and that both bilateral nephrectomy and bilateral ureter ligation suppress the response almost completely

The exact role of the kidney in red cell production is still not clear, however, it seems unlikely that the kidney actually controls red cell production by manufacturing or releasing the erythropoietic factor

Conclusion

In conclusion, it appears that the apparently simple [illegible] k mechanism that balances the body's need for [illegible] f hemoglobin is exceedingly [illegible] udies on the [illegible] However, [illegible] the employment of [illegible] the failure to distinguish between factors [illegible] multiplication and maturation of red cells and factors that control the rate with which stem cells are differentiated into pronormoblasts

Better and more standardized techniques and a clearer concept of the action of the erythropoietic factor should facilitate studies of this fascinating and challenging homeostatic mechanism

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SOME OBSERVATIONS ON THE STIMULATION OF ERYTHROPOIESIS BY HUMORAL FACTORS

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The regulatory mechanisms responsible for the normal erythroid stea state and the nature of the stimuli to erythropoiesis during periods of increa need have long attracted the attention of investigators. Until recent however, little was known concerning the manner in which erythroc equilibrium was maintained or restored. The endocrine glands, the nerv system, the spleen, hypoxia of myeloid elements, and a humoral factor ha all been implicated in the control of red blood cell production. Although t former three are capable of modifying erythropoiesis, they do not appear constitute the primary erythropoietic stimulus, whereas the role of arter oxygen tension as a basic determinant of erythropoietic activity has be widely confirmed by observations on experimental animals and human subjects However, the original theory that this effect was due to hypoxia of myeloid erythrocytic precursors has been disproved.

Carnot and Deflandre[1, 2] first described a humoral erythropoietic stimulat ing factor in 1906 and named this hypothetical substance hemopoietine In the course of research on the regeneration of organs, these investigators discovered that the serum of bled rabbits induced erythrocytosis when administered to normal rabbits In subsequent years this intriguing concept of a humoral erythropoietic regulatory mechanism was responsible for a number of attempts to confirm the existence of such an erythrocytogenic agent Some of these studies were in accord with those of Carnot and Deflandre, but many were in disagreement. The evident discrepancies in data that supported this hypothesis and the negative findings of other investigators were such that, prior to the past few years, the humoral control of erythropoietic activity was considered by most to be speculative and lacking in experimental support

Observations such as those of Reissmann[3] in 1950 on the stimulation of erythropoiesis in parabiotic rats maintained in a normal atmosphere following exposure of their partners to reduced oxygen te[illegible] ing large quantities of anem[illegible] poietic re[illegible] dem[illegible] B[illegible] ...s great[illegible] ...s On the basis of [illegible] ...istence of a humoral erythropoietic [illegible] no longer be denied However, the nature, site of production, and mode of action of the substances responsible for this effect have not yet been defined clearly

Our observations[9,10] *indicate that there are both thermostable and relatively thermolabile plasma erythropoietic factors that control, respectively,* the seemingly diverse physiological activities concerned with erythroblastic cellular division and the synthesis of hemoglobin. Extracts of active plasmas, which have been processed by boiling for 30 min, induce a singular type of erythropoietic response when administered to normal rats. This effect on erythropoiesis is characterized by erythrocytosis and reticulocytosis

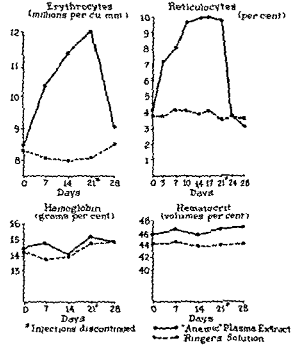
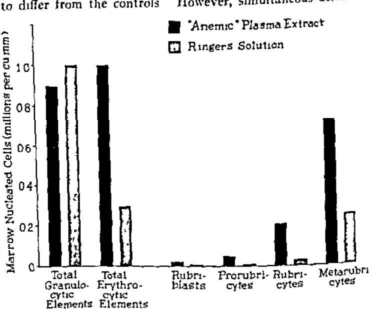


FIGURE 1. Erythrocytosis and reticulocytosis without increase in the hemoglobin or hematocrit values in normal rats given 18 daily injections (Sundays excepted) of boiled plasma extracts from rabbits made anemic by phenylhydrazine. Each injection was equivalent to 2 per cent of the recipient's body weight. Average determinations of 24 animals receiving the anemic plasma extract and a similar number of controls injected with Ringer's solution.

without associated increase in the hemoglobin or hematocrit levels (FIGURE 1). Myeloid erythrocytic hyperplasia involving a roughly proportional increase in all recognizable nucleated red cell precursors is also evident in the recipients of these anemic plasma extracts (FIGURE 2), and it provides, together with the erythrocytosis and reticulocytosis, conclusive evidence of increased erythropoietic activity. The newly formed cells responsible for the erythrocytosis are microcytic. Their small size is apparent on stained films and demonstrable graphically by Price-Jones measurements.[11,12] In our opinion,

this type of response is the result of accelerated erythroblastic cellular divisi without augmentation in hemoglobin synthesis.

Following discontinuation of the plasma extract injections, restoration normal erythrocytic equilibrium is prompt. Recent studies[12] have sho that this phenomenon apparently is due to impaired viability of the mic cytes produced in response to this particular stimulus. Quantitative pho colorimetric measurements of erythrocyte osmotic fragility in normal r injected daily for two weeks with a boiled anemic plasma extract were found to differ from the controls However, simultaneous determinati



... at the end of the injection ... There ... ecursors

by a direct cell enumeration technique employing red cell pipettes and hypotonic salt solutions as diluents demonstrated decreased osmotic resistance of the microcytes in the stimulated animals. Reversion to norma osmotic behavior accompanied the re-establishment of pretreatment erythrocyte counts and Price-Jones curves when the injections of plasma extrac were stopped. Since this fragility abnormality is not evident in situation involving increased activity of both humoral factors, it cannot be assume to be due to a specific action of the thermostable factor and most probabl results from alterations in cell size and/or shape, secondary to accelerate erythroblastic cellular division in the absence of a comparable stimulus fo

... boiling for 30 min. or more, whic ... is but fail to augment circulatin

hemoglobin or red cell mass, are also ineffective in enhancing the uptake of Fe^{59} in erythrocytes of recipient animals. However, the response to the same source materials, when tested in the unmodified state or after boiling for only short periods of time, is characterized by an increase in both hemoglobin and erythrocyte values and in iron-59 incorporation in hemoglobin. The effect of boiling on the activity of anemic plasma, as measured by the incorporation of Fe^{59} in the hemoglobin of nitrogen mustard-treated rats, is shown in TABLE 1. Although activity was demonstrable by this technique in recipients of the whole anemic plasma and of material boiled for 5 min., the [illegible]

[illegible]

globin formation is not

TABLE 1

EFFECT OF BOILING ON THE ERYTHROPOIETIC STIMULATORY ACTIVITY OF PHENYLHYDRAZINE-INDUCED ANEMIC RABBIT PLASMA AS MEASURED BY THE INCORPORATION OF Fe^{59} IN HEMOGLOBIN OF NITROGEN MUSTARD TREATED RATS

Mean of 4 Rats in Each Group ±1 Standard Deviation

Materials tested	Percentage Fe^{59} RBC uptake		
	18 hours	*24 hours*	*42 hours*
Unmodified anemic plasma	16 ± 2	19 ± 4	34 ± 5
Anemic plasma boiled 5 min	13 ± 2	20 ± 3	35 ± 2
Anemic plasma boiled 10 min	7 ± 3	10 ± 3	23 ± 4
Anemic plasma boiled 30 min	7 ± 2	9 ± 2	23 ± 4
Anemic plasma boiled 45 min	5 ± 2	6 ± 2	25 ± 3
Normal rabbit plasma	6 ± 2	9 ± 2	27 ± 5

We believe that these separable erythropoietic effects exerted by active plasmas are due to the presence of two factors that differ in nature and mode of action. One is characterized by an accelerated rate of erythroblastic cellular division and is related to a thermostable, ether-soluble fraction of plasma. The other, which involves hemoglobin synthesis, appears to be due to a relatively thermolabile, ether-insoluble factor.

The possibility that processing procedures might alter the chemical and physiological characteristics of a single factor must be considered, but appears quite unlikely. Ether extracts of unmodified anemic plasma evoke responses in recipient rats identical to those induced by boiled filtrates of such plasmas or their ether-soluble fraction. Furthermore, the demonstration of microcytes with decreased osmotic resistance in the blood of patients with polycythemia vera (FIGURE 3), a disorder associated with increased plasma erythropoietic activity,[9, 13, 14] and the retention in ether-insoluble fractions

of active plasmas of an enhancing effect on the incorporation of Fe^{59} in hemoglobin provide additional evidence against such an occurrence

We have yet to study an active plasma, regardless of the experimental or clinical conditions under which it was obtained, that did not possess activity attributable to both humoral factors Although their combined effects in normal recipients on hemoglobin synthesis and cellular division result in the production of increased numbers of normal cells, their properties are such that

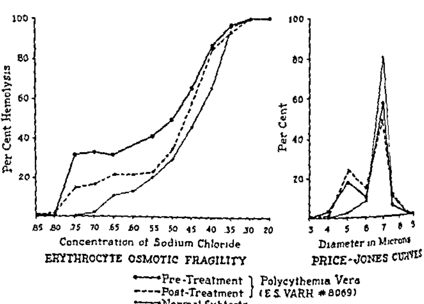


FIGURE 3 Erythrocyte osmotic fragilities determined by a direct cell enumeration technique and red cell diameter distributions (Price-Jones measurements) of a patient with

altered erythro-

The response

tions is charac-

, together with

myeloid erythrocytic hyperplasia. The presence of the relatively thermolabile factor can also be detected by the short-term method that utilize

the incorporation of iron-59 in hemoglobin as a means of demonstrating increased hemoglobin synthesis. Ether extracts of these same plasmas or their filtrates processed by prolonged boiling, however, contain only the factor that affects erythroblastic cellular division, and they do not enhance the formation of hemoglobin. Therefore, the differences in chemical and physiological attributes of the humoral erythrocytogenic agents would appear in many instances to explain the apparent disparity in experimental observations that have been described by various investigators.

The majority of our studies in regard to the chemical identities of the plasma erythropoietic factors have dealt with the thermostable, ether-soluble factor, and current information indicates that it is most probably a lipid. Similarities in the chemical and physical characteristics of batyl alcohol, the monoglycerol ether of *n*-octadecyl alcohol, and the ether-soluble plasma erythropoietic factor suggested a possible relationship between these two substances. Batyl alcohol was isolated from bovine yellow bone marrow by Holmes and his associates[16] in 1941 and a few reports have indicated that it possesses erythropoietic[16] and leukopoietic[17, 19] activity. Our observations[20] have confirmed the erythrocytic stimulatory effect of batyl alcohol, which was manifested in normal rats given daily injections of batyl alcohol in peanut oil over a period of four weeks by erythrocytosis due to microcytes with decreased osmotic resistance, reticulocytosis, and myeloid erythrocytic hyperplasia without increase in their hemoglobins or hematocrits. Batyl alcohol is also ineffective in enhancing the incorporation of Fe^{59} in hemoglobin of recipient animals.[21] On the basis of this erythrocytic response, which was identical in all respects demonstrable by the methods used to that observed following the administration of the thermostable, ether-soluble plasma erythropoietic factor, together with their common chemical and physical properties, it is suggested that batyl alcohol, originating in yellow bone marrow, may be identical with or closely related to this factor, perhaps as precursor material.

An increase in circulating thrombocytes has been a consistent finding in all animals to which we have given batyl alcohol with a prompt return to base line and control values when the injections are stopped.[20] Although daily doses of 25 mg. or less of batyl alcohol used in the above experiment did not alter the recipients' leukocyte counts, other studies[21] indicate that it also possesses granulopoietic stimulatory activity. However, greater amounts apparently are necessary to evoke this response in normal rats than are needed to elicit erythrocyte and thrombocyte increases.

Since certain lipid substances induce hemolysis which may be associated with thrombocytosis and leukocytosis, this possibility must be excluded before true myelopoietic stimulatory properties can be assigned to batyl alcohol. All of the hematological phenomena ascribed to batyl alcohol are compatible with a compensated hemolytic state with the exception of the erythrocytosis, and this could conceivably represent red cell cytoclasis with impaired viability of the resultant erythrocytic fragments. However, it would then be necessary to postulate definite augmentation in hemoglobin synthesis as a prerequisite for the maintenance of normal hemoglobin and

hematocrit values, and enhanced iron-59 incorporation in hemoglobin should be evident. The failure of batyl alcohol to increase erythrocyte iron 59 uptake in recipient rats[21] would appear to exclude hemolysis and supports the conclusion that this compound does exert a primary stimulatory effect on hematopoiesis

These observations on the erythropoietic, thrombopoietic, and probable granulopoietic activity of batyl alcohol are in accord with the theory that all aspects of myelopoiesis may be under the influence of humoral regulatory mechanisms Since erythrocytes, thrombocytes, and granulocytes are all derived from the multipotential myeloid reticulum cells, a single substance or activator-inhibitor complex may control the formation of all these hemic elements If this concept is confirmed by further investigation, the patho

thrombocytosis that accompany acute blood loss and certain types of intra vascular hemolysis and the increase in these hemic cells in patients wit polycythemia vera

The precise physiological and pathophysiological significance of th humoral erythropoietic factors is not known, but available data suppo the hypothesis that they constitute the primary erythropoietic stimulu Enhanced plasma activity has been found in a number of clinical and expe mental situations which, although of diverse etiologies, all with the exceptio of polycythemia vera have hypoxia as a common denominator Therefor it may be inferred that elaboration of the plasma erythropoietic factors probably dependent on the relationship between oxygen supply and tiss requirements at the as yet undetermined site or sites of their formatio Since some activity is demonstrable in the plasma of normal subjects, esp cially after concentration,[9, 22] it may be concluded that the humoral facto contribute to the maintenance of the normal erythroid steady state a probably constitute the primary erythropoietic stimulus.

A well-balanced equilibrium appears to exist that ensures, under norm conditions, an oxygen-carrying capacity of the blood commensurate w cellular needs Apparently this humoral regulatory mechanism is capable responding to even minor changes in the dynamic relationship betw oxygen supply and metabolic requirements. Erythropoietic activity there is altered accordingly, and equilibrium is re-established at the same o different level, as the situation warrants

The plasma factors are probably involved to some extent in all anem irrespective of their basic cause, but since erythropoiesis is dependent o

from the circulation Since there is no associated defect of the myel

ticulum, the marrow responds to this humoral stimulus by increasing the roduction of erythrocytes Anemia occurs only when erythrocytogenesis ils to keep pace with the peripheral loss Therefore, it is apparent that e anemias due to increased removal of red cells from the peripheral circulaon are intimately related to plasma erythropoietic stimulatory activity lthough the humoral agents *per se* are not of pathogenetic significance in iese anemic states, their increased elaboration would appear vital for the storation of normal values and in the case of persistent hemorrhage or emolysis equally indispensable to temper the severity of the anemic hypoxia. is possible that some failure or defect in the humoral regulatory control erythropoiesis may be responsible, at least in part, for the "aplastic ises" that have been described in some types of hemolytic disease, this ibject needs further study

The pathophysiological activity of the plasma factors in the anemias due to efective erythrogenesis is more speculative, but experimental observations ipport certain conclusions The stimulatory effect of erythropoietic factors resupposes adequate amounts of the various erythrocytic constituents gether with an intact myeloid reticulum It is evident that a normal iarrow response is otherwise precluded Studies on the effect of total body irradiation on plasma erythropoietic activity indicate that hematopoietic ements are not involved in the elaboration of the factors and that a regeneraye marrow is not a prerequisite for their formation[22] Plasma extracts om normal and irradiated rabbits rendered anemic by phenylhydrazine nd from those with anemia secondary to irradiation alone were each effective stimulating erythropoiesis in normal rats It follows that enhanced lasma activity secondary to anemic hypoxia should be demonstrable in the nemias that result from specific deficiency states or injury to myeloid eleients of known cause These conditions would include deficiencies of subiances such as vitamin B_{12} or iron, a reduction in myeloid erythrocytic recursors by physical or chemical means, displacement by foreign cells, or iversion of growth potential by leukemic transformation, and the hereditary emoglobinopathies Although the number of patients studied is as yet nall, increased activity has been found in each of these disorders Howver, the existent myeloid deficiency or defect prevents a normal marrow esponse to the humoral stimulus

It can be assumed that the plasma erythropoietic factors are not involved the actual production of the depressed erythroid values in these clearly efined anemias Nevertheless, their presence is probably essential for the ontinued attempt on the part of the marrow to produce some erythrocytes, nd they probably represent the mechanism by which normal erthyrocytic quilibrium is restored if the basic deficiency or defect can be corrected. he possibility exists, however, that some abnormality in the humoral rythropoietic regulatory mechanism involving impaired formation of the lasma factors, defective utilization, or both, may be of pathogenetic importance in certain other anemias of questionable or unknown cause Progress in this regard has been slow because of the difficulties associated with measuring relative degrees of activity

Polycythemia vera and secondary polycythemia are the hematologic disorders that, except for time factors, most closely simulate the imbalanc in erythrocytic equilibrium induced in normal rats by the administration the plasma factors Enhanced plasma erythropoietic stimulatory activit is demonstrable in patients with polycythemia vera and secondary poly cythemia, and has been a consistent finding in all of the patients with the disorders whom we have studied The overproduction of the plasma facto in patients with erythrocytosis due to decreased arterial oxygen saturation not surprising in view of the well-documented relationship between hypox and erythropoietic activity. Under these conditions, the hypoxic hypoxia undoubtedly the stimulus for the increased elaboration of the humoral facto which in turn transmit the need for augmentation of the oxygen-carryi capacity of the blood to the myeloid reticulum However, depending upo the severity of the arterial hypoxemia, the resultant increase in erythr poiesis may be insufficient to restore the normal relationship between oxyg supply and tissue requirements As a consequence, the physiological regul tory mechanism produces an unphysiological secondary polycythemia

Increased amounts of the humoral erythropoietic factors in patients wi polycythemia vera occur in the absence of demonstrable hypoxia. Since t factors are apparently not a product of hyperactive myeloid elements, t augmented plasma erythropoietic activity in this disorder supports the vi that polycythemia vera may be caused by a metabolic imbalance resulti in the production of excessive amounts of the plasma factors or, conversely, the failure to inactivate such materials at rates sufficient to maintain norm erythrocytic equilibrium Enhanced plasma activity should then persist these patients irrespective of the institution of specific myelosuppress therapy To date, we have studied the plasma of six patients with po cythemia vera after normal erythroid values had been achieved followi treatment with radioactive phosphorus Myeloid erythrocytic hyperpla (TABLE 2) was evident in the normal rats injected with ether extracts of plasma from each of these patients and was accompanied by erythrocyto and reticulocytosis

Augmented amounts of both the thermostable and relatively thermolat plasma factors have been demonstrated in polycythemia vera, thus explaini ... Howev-

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discernible during therapeutically induced remissions (FIGURE 3), a not unexpected finding in view of the persistence after treatment of enhanced erythropoietic activity as determined by *in vitro* plasma assays. Therefore,

These observations add further support to a causal relationship between the erythropoietic factors and polycythemia vera and are in agreement with the ... Berlin and his co-workers[24] of a bimodal red cell population in ... of erythrocytes with a normal life ... time of only a few days.

TABLE [illegible]
AVERAGE MARROW NUCLEATED CELLS PER CUBIC MILLIMETER OF RATS RECEIVING ETHER EXTRACTS OF PLASMAS FROM PATIENTS WITH POLYCYTHEMIA VERA IN THERAPEUTIC REMISSION

Source of plasma	Number of rats	Granulocytic elements	Erythrocytic elements
VARH—6738	3	1,059,125	1,280,000
VARH—8069	3	1,117,526	1,156,198
UMH—852687	3	1,008,570	1,096,517
UMH—772026	3	1,033,988	1,447,074
UMH—791448	3	862,802	1,065,074
UMH—811765	3	1,036,858	1,037,635
Normal subject	6	1,016,577	291,851

TABLE 3
AVERAGE THROMBOCYTE COUNTS (MILLIONS PER CUBIC MILLIMETER) OF RATS RECEIVING ETHER EXTRACTS OF PLASMAS FROM PATIENTS WITH POLYCYTHEMIA VERA

Source of plasma	Number of rats	Base line	1 week	2 weeks
Active disease				
UMH—880933	6	0.714	0.984	0.878
UMH—711033	6	0.676	0.900	0.832
UMH—876961	2	0.676	0.847	0.950
Therapeutic remission				
UMH—811765	6	0.759	0.938	0.966
UMH—852687	5	0.751	1.015	0.992
UMH—772026	6	0.755	1.188	0.940
UMH—791448	3	0.699	0.874	0.971
Normal subject	18	0.650	0.818	0.821
Ringer's solution	12	0.632	0.600	0.646

In view of the evidence that implicates the erythropoietic factors in the pathogenesis of polycythemia vera, experiments were designed to study the possible existence of humoral factors that might be responsible for the thrombocytosis and leukocytosis so commonly associated with this disease ... is in progress, but preliminary observations indicate that ... treated and untreated patients with ... normal rats

slight but definite increase in platelets has been a consistent finding in all recipients given such extracts daily for two weeks, with a prompt return to base line values after the injections were stopped. Although the normal unconcentrated human plasma extract was erythropoietically inactive, it ---- to contain minimal thrombocytic activity when the platelet ---- compared to those of rats injected ---- ution. Definitive conclusions in regard to these observation ---- additional study; however, they assume increased significance in the light of the apparent myelopoietic stimulatory effect of batyl alcohol, the evidence linking batyl alcohol with the thermostable, ether-soluble erythropoietic factor, and the findings that suggest that activity attributable to the latter factor may predominate in patients with polycythemia vera To date, all attempts to detect granulopoietic activity in polycythemic plasma extracts have been unsuccessful, but the possibility of varying degrees of sensitivity of myeloid elements to such a stimulus cannot be excluded

Summary

Our studies indicate that at least two humoral factors exert regulatory control over erythropoiesis A thermostable, ether-soluble agent stimulates erythroblastic cellular division, but does not augment hemoglobin synthesis, which appears to be governed by a relatively thermolabile, ether-insoluble factor

It is suggested, on the basis of similarities in their chemical and physiolog---- that the thermostable plasma erythropoietic factor and batyl ---- ----ounds ---- ----oietic activity of batyl alcohol, in addition to its effect on ---- ----pport the hypothesis that all aspects of myelopoiesis may be under the influence of humoral regulatory mechanisms Since erythrocytes, thrombocytes, and granulocytes are all derived from the multipotential myeloid reticulum cells, a single agent or activator-inhibitor complex may control the formation of all of these hemic elements Batyl alcohol, originating in yellow bone marrow, may be of primary importance in such a system

Although the precise physiological and pathophysiological significance of the plasma erythropoietic factors is not yet known, they most probably constitute the primary erythrocytogenic stimulus These humoral agents appear to contribute to the maintenance of the normal erythroid steady state and to be responsible for the increased erythropoietic activity in hypoxic and some types of anemic hypoxia

Observations on the erythropoietic and probable thrombopoietic activity of plasma from ---- vera indicate that the plasma factors are of et---- The stimulus to this derangement in ---- ----ains unknown

Further study of the humoral control of myelo---- should aid in clarifying the pathogenesis of certain obscure anemic states and hematological responses.

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STUDIES ON THE ACTIONS AND PROPERTIES OF THE CIRCULATING ERYTHROPOIETIC STIMULATING FACTOR*

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The concept of a circulating erythropoietic stimulating factor (ESF) has now been generally accepted. Evidence for its existence is derived from studies of the activity of blood serum and plasma obtained from animals rendered anemic by bleedings,[1-7] or hemolytic agents such as phenylhydrazine,[8-12] and from animals subjected to reduced barometric pressures[13-15] or treated with erythropoietic metals such as cobalt.[16]

In animals supplied with adequate quantities of organic and inorganic nutrients necessary for the normal production of red cells and with an undamaged responsive bone marrow, it has been proposed that the oxygen supply to the tissues determines the rate of formation of red cells.[19] In this regard, a decrease in the oxygen content of the arterial blood, manifested either as a decrease in oxygen tension (hypoxic hypoxia) or as a lowering in oxygen-carrying capacity (anemic hypoxia), may stimulate a hypothetical oxygen-sensitive erythropoietic center(s) to elaborate and release the ESF. Erythropoietic metals including cobalt may operate through histotoxic hypoxia by inhibiting certain oxidative enzyme systems, including choline oxidase, succinic dehydrogenase, cytochrome oxidase, and catalase.[17]

This paper will describe (1) a critique of the methods employed for evaluating the ESF, (2) the erythropoietic effects of plasma and urine extracts obtained from patients with Cooley's and hypoplastic anemia, (3) attempts at purification of human urinary ESF, and (4) studies of blood formation in isolated perfused hind limbs of rats.

erythropoietic activity, unless simultaneous measurements are made of the red cell numbers and hematocrit values, or preferably the total circulating red cell volume, a rise in the numbers of reticulocytes may be the consequence of an anemia-inducing action of the agent being tested Employed alone, hematocrit determinations also may not be critical, since it is possible to envision the hematocrit values remaining unchanged or even decreased in the face of erythropoietic stimulation involving the production of smaller sized cells Similarly, hemoglobin concentrations may be augmented, not as a result of increased red cell production, but as a consequence of the formation of cells with increased hemoglobin content The employment of Fe^{59}-incorporation into the circulating red cells or desoxyribonucleic acid (DNA) incorporation in spleen or marrow as measures of increased erythropoiesis is subject to the same criticism as the reticulocyte counts alone Moreover, increased Fe^{59}-incorporation may occur as a result of the ability of the agent tested to cause a decrease in the circulating iron pool, thus permitting a greater proportionate uptake of the isotope [15] In short, although single parameters such as reticulocyte counts and Fe^{59}-incorporation may serve as useful indexes for initial screening procedures, it is only through a detailed analysis of the peripheral blood picture and the blood-forming organ response that a true evaluation of the erythropoietic effects of any agent can be made

Our studies have employed the alterations in the peripheral red cell numbers, the hemoglobin, hematocrit, and reticulocyte values, and also the numbers of the nucleated red cells in the bone marrows of intact rats as the criteria for erythropoietic stimulating activity Other investigators[11, 19] have used hypophysectomized animals as recipients, presumably because of their greater sensitivity to blood-stimulating factors However, caution should be applied in the interpretation of the results from these animals because of their sensitivity to the erythropoietic stimulating actions of small amounts of adrenal or androgenic steroids[20] that might be present in the plasmas or in sera obtained from hypoxic animals Similarly, the use of starved animals as recipients may be open to question in view of their sensitive erythropoietic response to small amounts of nonspecific nutrients that may be present in plasma or extracts derived from it

In this connection, we have recently found* that acidified boiled filtrates of plasma obtained from rabbits given a single large dose of inorganic cobalt markedly stimulate Fe^{59}-incorporation into the red cells of starved rats In these studies each of 60 rabbits averaging 3 5 kg was administered a single dose of $CoCl_2 \cdot 6H_2O$ amounting to 50 to 70 mg kg body weight Twelve to 24 hours later the rabbits were exsanguinated and the heparinized plasma was brought to pH 5 5 by the addition of 1 N HCl The plasma was added to sterile boiling physiological saline and the mixture maintained at 70 to 80° C in a liquid petrolatum bath for 10 min The plasma was filtered, the filtrate obtained was cloudy and dark brown in color and appeared to

* Winkert and Gordon, unpublished

light-straw color, and approximately 10 per cent more dilute than the starting plasma.

The bio-assay procedure was a modification of that devised by Goldwasser *et al.*[21] Mature female rats of the Long-Evans strain were used as test animals. They were starved for 4 consecutive days. Subcutaneous injections of the plasma filtrates (3 ml. of cobalt plasma and 3.3 ml. of normal plasma) were begun on the second day, 24 hours after the commencement of starvation. Injections were continued on the third and fourth days of starvation. At 3 hours after the last plasma filtrate injection, 1.0 μc of Fe^{59} in 0.2 ml. of solution was injected intravenously. This contained 0.5 μg of iron as ferrous citrate plus 20 μg of iron as $FeCl_3 \cdot 6H_2O$ added as a carrier. Twelve hours later, counting rates were determined for the saline-washed red cells. Reticulocyte counts also were made from undiluted blood, using Brecher's

TABLE 1
EFFECTS OF PLASMA FILTRATES FROM COBALT-TREATED AND UNTREATED RABBITS ON RETICULOCYTE NUMBERS AND Fe^{59} INCORPORATION IN RED CELLS OF STARVED RATS
(Means ± Standard Errors)

	Retics (percentages)	Cpm/ml. RBC	Loss in body wt. (percentages)
Cobalt-treated plasma filtrate	1.2 ± 0.2	768 ± 22	38
Normal plasma filtrate	0.5 ± 0.1	89 ± 7	34
Significance	$p < 0.01$	$p < 0.05$	—

method.[22] The results in TABLE 1 indicate that, with the method employed, [illegible]

It was felt that if this plasma truly possessed erythropoietic stimulating properties, it should be capable of inducing polycythemic effects when injected chronically into intact rats. Accordingly, 11 daily subcutaneous injections of the same plasma filtrates, in dosages similar to those employed above, were administered to adult female rats of the Long-Evans strain. Reticulocyte determinations were made on the sixth day and red cell counts, hematocrit, and hemoglobin determinations, on the twelfth day (24 hours after the last injection). It may be noted from TABLE 2 that no significant differences were observed in the effects of plasma filtrates obtained from cobalt-treated rabbits and those from normal rabbits when intact fed rats were utilized as the recipients.

TABLE 2
EFFECTS OF PLASMA FILTRATES FROM COBALT TREATED AND UNTREATED RABBITS ON ERYTHROCYTIC PARAMETERS OF INTACT FED RATS (Mean Values)

	Retics (percentages)		RBC (million cu mm)		Hb (gm percentages)		Hct (percentages)		Body wts (gms)	
	B	A	B	A	B	A	B	A	B	A
Cobalt-treated plasma filt	2 4	3 8	9 1	8 9	16 4	16 7	46 6	48 8	187	200
Normal plasma filt	2 5	3 8	9 3	9 3	16 4	16 7	47 6	48 1	181	190

B = Before injections, A = After injections

TABLE 3
EFFECTS OF WHOLE PLASMA FROM COBALT-TREATED RABBITS ON ERYTHROCYTIC PARAMETERS OF INTACT RABBITS (Mean Values)

Retics (percentages)		RBC (million/cu mm)		Hct (percentages)		Body wts (kg)	
B	A	B	A	B	A	B	A
3 9	2 7	5 5	3 8	31 8	25 3	3 7	3 3

B = Before injections, A = After injections

no erythropoietic stimulation occurred in rabbits injected with the whole untreated cobalt plasma On the contrary, a decrease in all 3 erythrocytic parameters was evident, probably due to the loss in weight and decrease in food intake exhibited by the animals during the course of the experiment

It is thus clear that, although plasma obtained from cobalt-treated rabbits stimulates erythropoiesis in starved rats, it is incapable of doing so in fed animals Chemical analyses indicate a considerably greater nitrogen content in the plasma filtrates obtained from the cobalt-treated rabbits than in those from untreated control rabbits The nitrogenous substances possibly represent products of tissue destruction induced by the large dose of cobalt Therefore, the effects observed with plasma filtrates of the cobalt-treated rabbits may be due to contained, non-specific nutrient factors to which the starved[23] or hypophysectomized[24] rat responds sensitively, rather than to the presence of the ESF It is equally possible that the erythropoietic stimulating effects observed with the cobalt plasma filtrates are not due to an energy contribution, in view of the relatively small amounts of material administered Evidently, the substance(s) present in such filtrates is capable of mobilizing endogenous materials for prompt fabrication of red cells in the

starved, but not in the fed, animal. It would be important to determine the nature of this substance and why the state of inanition permits this action to occur.

Presence of ESF in Extracts of Plasma Obtained from Patients with Cooley's and Hypoplastic Anemia

Work in our laboratories[26, 27] has established the presence of large quantities of ESF in the plasma of some children with Cooley's and hypoplastic anemias. Transfusion of such patients with whole blood or packed red cells results in a rapid disappearance of the ESF from the plasma. Two representative cases of each of these anemias are provided below.

Case 1. F. D. was born in June 1950 and was anemic from the age of 1 year; diagnosis of Cooley's anemia was made at 18 months of age. He has required transfusions approximately every 3 weeks for the past 2 years. The patient is sensitized to the Rh_0 and Kidd blood group factors and has not been splenectomized. Physical examination revealed an underdeveloped boy weighing 45 lb. with a large abdomen and typical facies associated with marked Cooley's anemia. The spleen is huge, reaches below the brim of the pelvis, and extends over into the right lower quadrant. The liver is also enlarged and its lower edge reaches the level of the umbilicus. On October 18, 1956, prior to transfusion, the blood count was as follows: Hb 5.0 gm. per cent, RBC 2.3 million/cu. mm., WBC 5800/cu. mm., platelets 75,000/cu. mm., reticulocytes 1.5 per cent, differential count, band forms 27 per cent, segmented polys 37 per cent, lymphocytes 31 per cent, monocytes 4 per cent, eosinophils 1 per cent, 4 nucleated RBC/100 WBC. The patient received the following transfusions of Group O Rh-negative, Kidd-negative compatible blood: on October 18, 250 ml. whole blood; on October 19, 500 ml.

reticulocytes seen, differential count band forms 4 per cent, segmented polys 40 per cent, lymphocytes 52 per cent, monocytes 2 per cent, eosinophils 2 per cent Patient received the following transfusions of Group A, Rh-positive compatible blood on January 22, 500 ml whole blood, on January 23, 500 ml whole blood, on January 24, 500 ml whole blood Following transfusions, on January 25, 1957, the Hb was 11 0 gm per cent and the RBC 4 5 million /cu mm.

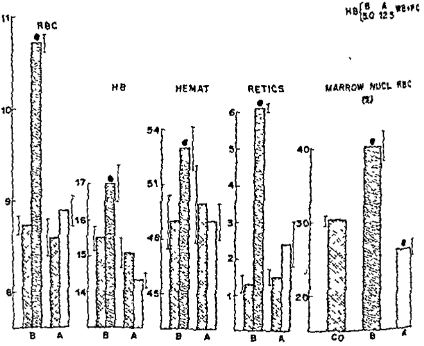
Blood obtained from the patients was heparinized and centrifuged, the plasma was acidified to pH 5 5 and boiled for 10 min , as described previously[4, 8-10] Saline was added during the boiling procedure in order to maintain a volume equal to that of the original plasma Following filtration, the residues were discarded and the filtrates were neutralized with 1 N NaOH

Groups of 5 normal adult female rats of a modified Long-Evans strain weighing 180 to 200 gm were injected daily for 8 days with 2 3 to 3 5 ml of the plasma filtrates obtained before transfusion and following the last transfusion The volumes of filtrates were equivalent to 3 ml of the original untreated plasma Hematological determinations were conducted in the recipient rats before and after the experimental treatment with the plasma extracts Red cell counts were made in duplicate and were required to agree within ±5 per cent Hemoglobin concentrations were determined by the acid hematin method with a photoelectric colorimeter Hematocrit values were estimated by a capillary tube method, utilizing centrifugal speeds corresponding to 11,000 rpm for 5 min Reticulocytes were counted from dried smears stained with new methylene blue[22] One thousand to 1500 RBC were enumerated, and the reticulocytes expressed as a percentage of these. At the end of the experimental period of injections, the rats were exsanguinated, the femurs quickly dissected, and the marrow contents prepared in suspension form in a small quantity of homologous serum Smears made from these suspensions were stained with the Ralph benzidine technique[23] and counterstained with Wright's The benzidine method permits a more precise estimation of the hemoglobin-bearing red cells (erythroblasts and normoblasts) The nucleated RBC values of marrow are presented as a percentage of 1500 to 2000 nucleated cells counted in the smears of each recipient animal

Figures 1 and 2 show strong erythropoietic stimulating activity in the boiled plasma filtrates of the patient with Cooley's anemia and the one with hypoplastic anemia, just prior to transfusion when their hemoglobin values were 5 0 gm per cent and 4 5 gm per cent, respectively The recipient rats experienced significant increases in red cell count, hemoglobin, hematocrit, and reticulocyte values, and in the percentages of nucleated RBC within the femoral marrows It is of interest to note that strong erythropoietic stimulating activity is not exhibited consistently in the patient with hypoplastic anemia On a previous occasion[25] filtrates prepared from his plasma, when his pretransfusion hemoglobin level was 5 gm per cent, were virtually inactive.

Figures 1 and 2 also indicate that transfusion to high hemoglobin levels with whole blood or packed red cells caused a lowering in the quantity of the

ESF in the plasma of the patients studied, in most cases to nondetectable levels. This situation is reminiscent of the rapid curtailment of erythropoietic activity in animals subjected to transfusion polycythemia.[11] Evidently, the reduction in the intensity of the anemia and associated hypoxia by the transfused cells is an effective procedure for suppressing the production



... (11b) hemato- ...d RBC ...) with ...BC per ...s repre- ...ecipient ...different ...indicate ...after (4) ...patient ...PC) are

of the ESF. Transfusion may operate by increasing oxygen supply to the tissues or to the site of production of the ESF. In addition, the speed at which the disappearance of erythropoietic activity occurs in the patients would imply a rapid utilization or elimination of the increased amounts of the factor present in the plasmas of anemic subjects, a point in agreement with animal studies.[13, 30]

Somewhat surprising was the finding that considerable amounts of the ESF were detected in the plasma of a child with chronic aregenerative anemia, since previously[45] no significant activity was present This difference occurred despite a relatively similar hematological picture of the patient at the time the pretransfusion specimens were obtained It is now clear from

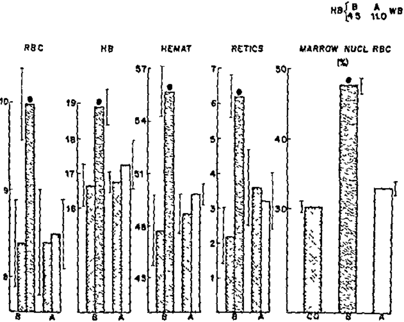


FIGURE 2 Case 2 mean peripheral red cell numbers (RBC) hemoglobin (Hb), hematocrit (Hemat), and reticulocytes (Retics) response, as well as bone marrow nucleated RBC [illegible]

before (B) and after (A) transfusion are compared to the control (CO) values Hemoglobin values of the patient before (B) and after (A) transfusion with whole blood (WB) are indicated at upper right Reproduced by permission from *Acta Haematologica* [37]

work performed in our laboratories that fluctuations in the quantity of the ESF also may occur in the blood of anemic animals, even though the procedures employed to induce the anemia were essentially the same Such variations in the amount of the ESF, in both anemic patients and animals, could result conceivably either from differences in its rate of production, its utilization by the blood-forming tissues[40] and destruction by the liver,[7, 31] or in kidney threshold for the factor at the time of blood sampling

In the present study, erythromicrocytosis reported previously[6,23,27] was not a consistent feature of the response to the plasma extracts. Thus, although calculations indicated that the mean corpuscular volumes and mean corpuscular hemoglobin values were reduced in the rats receiving filtrates of pretransfusion plasma of Case 1, they were unchanged in Case 2. The possibility that different degrees of iron absorption and assimilation exist in rats receiving the different filtrates should be considered. Also, it is conceivable that the different filtrates possess varying proportions of the 2 factors suggested by Linman and Bethell:[33] (1) thermostable, concerned with erythroblastic cellular division and (2) thermolabile, involved in hemoglobin synthesis.

Presence of the ESF in the Urine of Anemic Subjects

2 patients, as well as in others, ine. Seventeen 24-hour urine and 1 anemic adult with hemo Four additional samples wer bin levels. All the urines wer preserved at ... d per liter. Five of the urin samples from patients with Cooley's anemia and 1 from a normal child wer subjected to the acidification-boiling procedures described previously[6,1] The remainder of the urine samples were tested without modification.

As with the plasma samples described above, normal mature female ra of a modified Long-Evans strain weighing 150 to 200 gm. were used f testing the urines. Four or 5 rats were employed for each assay. Each r received subcutaneous injections of 3 ml. urine once daily for 10 to 20 da Reticulocyte, red cell, hemoglobin, and hematocrit determinations w performed prior to the injection period. Reticulocyte counts were m again on the sixth day and red cell, hemoglobin, and hematocrit estimati were repeated on the day following the last injection. In 4 assays, b marrow smears were prepared after the last injection, following the met described above. In 1 assay, blood volume determinations were perfor according to the method of Piliero and Pansky.[35]

Of 5 urine samples obtained from the patients with Cooley's anemia ... only the sample colle (F. D., above) The other 4 inac urine from a no boy of the same age was ineffective (Normal 1, TABLE 4).

The remaining 12 urine samples obtained from the anemic children the adult with a benign thymoma were not acidified and boiled. Eig these evoked increases in some of the erythrocytic values. These incl 2 of the 4 samples obtained from the Cooley's patient (F. D., Cooley's an 2, 3 in TABLE 4), 3 of the 5 samples taken from the hypoplastic anemia su (R.C., hypoplastic anemia 1,2,3) and one each from a case of myelog leukemia (myelogenous leukemia 1), benign thymoma, and sickle cell an

However, only 4 of the 8 positive responses involved more than 2 erythrocytic parameters

No erythropoietic stimulating activity was noted in a Cooley's patient's urine (T D, Cooley's anemia, transfused 1, TABLE 4) after a transfusion had brought his hemoglobin level to 12 gm per cent Similarly, the urine from the myelogenous leukemia patient in remission (myelogenous leukemia 2, TABLE 4) was inactive, as was the urine from the normal girl (Normal 2)

TABLE 4

EFFECTS OF URINE FROM ANEMIC PATIENTS ON PERIPHERAL BLOOD OF THE RAT[a,b]

Status of Donor				Effects in recipient rats (mean ± S E)				
Age (yr)	Diagnosis		Hemoglobin (gm %)		RBC (millions/cu mm)	Hemoglobin (gm %)	Hematocrit (percentages)	Retics (percent ages)
6 ♂	Cooley's anemia	(1)	5.4	B[c]	9.1 ± 0.2	16.0 ± 0.5	47.2 ± 1.0	2.1 ± 0.2
				A	12.0 ± 0.4**	18.4 ± 0.8*	54.6 ± 2.2*	4.6 ± 0.8*
		(2)	5.5	B	8.7 ± 0.4	15.3 ± 0.4	46.1 ± 1.3	2.5 ± 0.9
				A	9.7 ± 0.2	17.7 ± 0.5*	51.7 ± 2.6	7.7 ± 0.8**
		(3)	4.5	B	9.0 ± 0.2	14.9 ± 0.2	46.9 ± 1.9	3.9 ± 0.7
				A	11.1 ± 0.6*	18.3 ± 0.9*	57.7 ± 2.1*	8.3 ± 1.3*
	Cooley's (transfused)	(1)	12.0	B	9.5 ± 0.1	17.6 ± 0.3	50.0 ± 1.7	2.9 ± 0.3
				A	9.0 ± 0.4	14.8 ± 0.2	44.0 ± 1.0	3.3 ± 0.4
7 ♂	Hypoplastic anemia	(1)	5.5	B	8.5 ± 0.1	14.7 ± 0.2	45.6 ± 1.2	3.0 ± 0.4
				A	11.0 ± 0.1**	18.1 ± 0.7**	55.6 ± 1.4**	5.2 ± 0.4**
		(2)	4.5	B	8.7 ± 0.3	15.5 ± 0.6	46.1 ± 1.2	1.9 ± 0.4
				A	10.3 ± 0.3**	17.1 ± 0.1*	51.0 ± 0.7**	7.7 ± 0.9**
		(3)	6.0	B	9.0 ± 0.2	15.9 ± 0.3	49.8 ± 0.9	2.9 ± 0.8
				A	10.0 ± 0.2*	16.0 ± 0.7	50.3 ± 0.7	3.7 ± 0.7
3 ♂	Myeloblastic leukemia	(1)	6.5	B	9.3 ± 0.2	14.5 ± 0.4	54.5 ± 0.3	3.1 ± 0.6
				A	11.6 ± 0.2**	16.5 ± 0.2**	57.0 ± 2.1	6.0 ± 0.4**
	Myeloblastic leukemia in remission	(2)	11.7	B	8.3 ± 0.1	15.6 ± 0.9	45.0 ± 4.3	2.9 ± 1.3
				A	8.6 ± 0.1	14.9 ± 0.6	42.0 ± 1.0	3.2 ± 0.0
5 ♀	Sickle cell anemia		6.5	B	9.0 ± 0.2	14.9 ± 0.2	49.5 ± 0.5	2.0 ± 0.4
				A	10.2 ± 0.3*	16.5 ± 0.5*	52.0 ± 1.4	2.7 ± 0.9
68 ♀	Benign thymoma		7.0	B	9.1 ± 0.1	15.6 ± 0.4	50.4 ± 1.3	2.8 ± 0.7
				A	10.2 ± 0.1*	16.7 ± 0.3	51.9 ± 1.1	4.6 ± 0.6
4 ♂	Normal	(1)	13.5	B	9.2 ± 0.2	16.6 ± 0.6	49.6 ± 1.2	2.4 ± 0.3
				A	8.9 ± 0.2	16.6 ± 0.6	45.4 ± 2.2	4.7 ± 0.7*
5 ♀	Normal	(2)	11.0	B	8.3 ± 0.1	15.6 ± 0.3	49.6 ± 0.9	3.0 ± 0.8
				A	8.2 ± 0.2	14.9 ± 0.7	46.5 ± 1.1	3.2 ± 0.4

[illegible] Experimental Biology and Medicine

The 2 samples of urine collected from the boy with hypoplastic anemia that evoked the marked increases in all 4 peripheral parameters listed in TABLE 4 (hypoplastic anemia 1 and 2), also evoked erythroid marrow hyperplasia, as did one sample taken from the Cooley's anemia patient (Cooley's anemia 2, TABLE 5) Blood volume values, determined in the animals receiving urine sample 1 of the hypoplastic anemia patient, were significantly elevated (TABLE 6) Nucleated red cell percentages were increased in the

TABLE 5
EFFECTS OF URINE FROM ANEMIC PATIENTS ON BONE MARROW OF THE RAT[a]

Status of donor			Marrow response after treatment (mean ± S E)
Age	Diagnosis	Hb (gm percentages)	Nucleated RBC (Percentages of total nucleated cells)
6 ♂	Cooley s anemia (1)	5 4	36 6 ± 3 4
	(2)	5 5	42 2 ± 6 9*
7 ♂	Hypoplastic anemia	5 5	45 8 ± 1 9†
		4 5	44 2 ± 4 0†
3 ♀	Sickle cell anemia	6 5	27 2 ± 7 0
6 ♂	Normal	11 5	41 0 ± 1 5*
	50 untreated control rats		32.4 ± 2 4

* Mean significantly greater than untreated controls, $p < 0.05$
† Mean significantly greater than untreated control, $p < 0.01$.
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TABLE 6
EFFECT OF URINE FROM AN ANEMIC PATIENT ON BLOOD VOLUME OF THE RAT[a]

Donor	Blood volume (cc /100 gm body wt)
Hypoplastic anemia (Hb 5 5 gm per cent)	10 1 ± 0 3*
Untreated controls	8 3 ± 0 4

* Mean significantly greater than untreated control, $p < 0.01$.
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Analysis of the results indicated that only 25 per cent of the 17 un[illegible] centrated urine samples taken from 7 anemic humans displayed ery[illegible] [illegible] ...dence of act[illegible] urine than [illegible] unconcent[illegible] work by M[illegible] nal human [illegible] ng suggests [illegible] stant source of erythropoietically active material. It is probably more [illegible]

* Personal communication

since more injections are generally required to evoke detectable responses However, as a source for the isolation of the factor, urine has the advantage of being readily available in large quantities relatively free of protein It is not certain why urine is less frequently active than plasma Examination of the clinical records indicates no correlation between the presence of activity in urine and the general status of the patient, the severity of the anemia,* or the degree of concentration (specific gravity) of the urine In this latter regard, the question of normal and pathological variations in renal threshold should be studied

Partial Purification of the Urinary ESF

Concentration of the ESF in plasma has been accomplished by some workers Rambach *et al*[12] have indicated that the plasma ESF is a mucoprotein or a substance associated with mucoprotein Along the same lines, we have recently shown[16] the presence of a metachromasia reaction when the cationic dye toluidine blue O is added to filtrates of plasma from human and rabbit sources possessing erythropoietic stimulating activity Since human urinary gonadotropin is acknowledged to be glycoprotein in nature and since Albert,[16] after utilizing a number of procedures, concluded that kaolin is well suited as an adsorbent for the concentration of this hormone from urine, the present experiments were conducted to determine whether a concentration of the urinary ESF could be achieved by adsorption on kaolin Of interest in this connection are the observations of Marcotte-Boy *et al*[17] that increased amounts of mucoprotein are detectable in the urine of rabbits subjected to repeated bleedings or to lowered barometric pressures

First Kaolin Experiment

The first experiment employed kaolin in a series of batch operations with successive adsorptions at lower pHs and successive elutions at increasing pHs Each eluate was dialyzed separately, neutralized with 1 N HCl, and combined with the others Although it was realized that this type of procedure does not define the most efficient manner in which to use kaolin (that is, it does not indicate the best adsorption pH, the minimal quantity of eluate obtainable or the best elution pH), it should indicate, employing a minimum of urine, whether kaolin is suitable at all

Urine from a 6-year-old boy with Cooley's anemia (hemoglobin 4 0 gm per cent at the time the 24-hour urine sample was obtained) was used as the starting material The urine sample was distinctly active when tested in adult intact female rats given subcutaneous injections daily for 13 days of 3 ml of the filtered, but otherwise untreated urine The results are presented in TABLE 7

Method Six hundred and eighty-five ml of urine was mixed with 10 gm of kaolin (Fisher K-5 acid-washed, American standard kaolin) at pH 6 2 (pH of original urine) Mixing was conducted at 5° C, using a large pipette

* We have found more recently that urine samples obtained from severely anemic patients (Hb levels below 4 0 gm per cent) are almost invariably active

TABLE 7
ERYTHROPOIETIC EFFECTS OF UNTREATED COOLEY'S URINE USED FOR FIRST KAOLIN EXPERIMENT IN NORMAL RATS (Means ± Standard Errors)

	RBC (million cu mm)	Hb (gm percentages)	Hct (percentages)	Retics (percentages)	Body wts. (gm)
Initial	9 0 ± 0 2	14 9 ± 0 02	46 9 ± 1 9	3 9 ± 0 7	164 ± 6
Final	11 1 ± 0 6	18 1 ± 0 9	57 7 ± 2 3	8 3 ± 1 3	184 ± 7
p values	<0 02	<0 02	<0 02	<0 02	—

TABLE 8
EFFECTS OF KAOLIN ELUATES OF COOLEY'S URINE ON ERYTHROPOIESIS IN NORMAL RATS (Means ± Standard Errors)

	Hb (gm percentages)	Hct (percentages)	Retics (percentages)	Body wts. (gm)
Kaolin eluates				
Initial	15 5 ± 0 5	45 3 ± 1 3	3 1 ± 0 2	190 ± 9
Final	17 2 ± 0 3	52 5 ± 2 2	7 5 ± 0 2	193 ± 9
p values	<0 05	0 05	<0 01	—
4 untreated controls				
1st day	15 7 ± 0 7	47 3 ± 2 0	3 0 ± 0 1	208 ± 4
11th day	15 8 ± 0 3	46 4 ± 0 6	2 8 ± 0 1	210 ± 2

The slurry was centrifuged and the supernatant poured off and brought to *p*H 5 2 with 1 N HCl This was added to 10 gm of fresh kaolin.

The material adsorbed at *p*H 6 2 was eluted separately at *p*H 8 9 and 10, employing 20 ml of ammonium acetate buffer twice at each *p*H The final elution was carried out with 1 M NH_4OH The material adsorbed at *p*H 5 2 was eluted similarly All the *p*H 8 eluates were combined (80 ml) and dialyzed for 36 hours against 1 liter of 0 9 per cent saline The *p*H 9, *p*H 10, and 1 M NH_4OH elutions were treated similarly Finally, all elutions were neutralized and combined, about 300 ml of total eluate was obtained The material was assayed by administering 2 daily 3-ml subcutaneous injections to each of 4 intact rats for 10 days Only 3 parameters were used to assess the activity The results are shown in TABLE 8

Second Kaolin Experiment

Since the first experiment demonstrated that active material could be adsorbed on and eluted from kaolin, a second run was conducted to determine an optimal *p*H for adsorption

Method One hundred ml of urine from a Cooley's patient (Hb 4 5 gm per cent, see TABLE 9 for original assay) was acidified with 1 N acetic acid to *p*H 6 2. A second 100 ml was brought to *p*H 5 2, a third to *p*H 4 8, and a fourth to *p*H 4 3 Each of the 4 samples was added to 1 5 gm of kaolin,

hile mixing up and down with a pipette. Each of these 4 kaolin-urine nixtures was centrifuged, resuspended in a smaller volume of supernate, ombined, and recentrifuged. The supernates corresponding to each adsorpion pH were kept separately. Each kaolin cake was then suspended in 6 ml f ammonium acetate buffer, mixed with a pipette, and centrifuged. This as repeated with another 6-ml portion of the same buffer.

TABLE 9
ERYTHROPOIETIC EFFECTS OF UNTREATED COOLEY'S URINE USED FOR SECOND KAOLIN EXPERIMENT IN NORMAL RATS (Means ± Standard Errors)

	Hb (gm percentages)	Hct (percentages)	Retics (percentages)	Body wts (gm)
Initial	16 9 ± 0 2	49 3 ± 1 8	2 3 ± 0 4	201 ± 6
Final	19 3 ± 0 9	60 4 ± 2 2	8 0 ± 0 4	209 ± 4
p values	<0 05	<0 01	<0 01	

Four rats given daily subcutaneous injections for 13 days of 3 ml filtered untreated rine, reticulocyte counts determined after seventh injection

Ammonium acetate buffers corresponding to *p*H 8, *p*H 9, and *p*H 10, and lso 1 M NH_4OH and 2 M NH_4OH, were used successively for elution. The

om a burette to 1 M NaH_2PO_4 until a *p*H of 6 5 was attained. The solution as supersaturated and had to be warmed to be kept from precipitating. 150 l. of this stock buffer solution was then added to 1350 ml of distilled water inal *p*H 6 8). This diluted buffer was used for dialysis and for the buffer ontrol. The same procedure was followed for the *p*H 9, *p*H 10, 1 M NH_4OH, nd 2 M NH_4OH eluates.

The *p*H 8, *p*H 9, *p*H 10, 1 M NH_4OH, and 2 M NH_4OH eluates from the H 6 2 adsorptions were all combined after dialysis. The same procedure as followed for the eluates from the *p*H 5 2, *p*H 4 8, and *p*H 4 3 adsorptions. t the time the eluates were combined they were all found to be at about *p*H 8. Approximately 60 ml of the combined eluates was obtained for each f the 4 adsorptions.

The eluates were tested in a short assay employing reticulocyte and hematont determinations. Four groups of 4 intact adult female rats were given daily 3-ml injections subcutaneously and examined on the fifth day. Four ontrols received 3-ml injections of the buffer for the same length of time. s adjudged from the reticulocyte response, it may be observed that the rinary ESF was adsorbed on kaolin at all 4 *p*Hs employed (TABLE 10). hus, it may be concluded from this second experiment that, unlike the uman urinary gonadotropin, the adsorption of the urinary ESF is relatively ndependent of *p*H in the *p*H range of 4 3 to 6 2.

TABLE 10
EFFECTS OF KAOLIN ELUATES CORRESPONDING TO 4 ADSORPTION pHs ON ERYTHROPOIESIS IN NORMAL RATS (Means ± Standard Errors)

Eluates injected	Hct (percentages)	Retics (percentages)
Adsorbed at *p*H 6 2		
Initial	48 7	2 8 ± 0 1
Final	49 1	5 8 ± 0 4 $(p < 0\ 01)$
Adsorbed at *p*H 5 2		
Initial	49 0	3 0 ± 0 5
Final	48 1	6 6 ± 0 4 $(p < 0\ 01)$
Adsorbed at *p*H 4 8		
Initial	47 7	2 5 ± 0 6
Final	49 2	6 7 ± 0 3 $(p < 0\ 01)$
Adsorbed at *p*H 4 3		
Initial	48 3	2 6 ± 0 6
Final	47 0	6 2 ± 0 5 $(p < 0\ 01)$
Buffer control		
Initial	49 6	2 5 ± 0 3
Final	43 8	2 4 ± 0 2

Third Kaolin Experiment

From the previous experiment, it was determined that *p*H 4 8 was as g as, if not better than, the other *p*Hs for adsorption of the urinary fact It was the purpose of the third kaolin run to determine the best elution *p* Unlike the previous experiment, in which separate batches were adsorbed different *p*Hs, a single batch was used and eluted successively with hig *p*H solutions (gradient elution)

0 9 per cent saline again for 48 hours All eluates were approximately neutral, reading about *p*H 6 8

Six groups of adult female rats weighing 170 to 240 gm and averaging 185 to 195 gm per group were used for the assay Four rats comprised each group Each rat of the 6 groups received 3-ml subcutaneous injections of the following materials daily for 5 days Group I, sterile 0 9 per cent saline; Group II, *p*H 8 eluate in 0 9 per cent saline, Group III, *p*H 9 eluate in 0 9 per cent saline, Group IV, *p*H 10 eluate in 0 9 per cent saline, Group V, 1 M NH_4OH eluate, neutralized, in 0 9 per cent saline, Group VI, 2 M NH_4OH eluate, neutralized, in 0 9 per cent saline Reticulocyte counts and hematocrit determinations were made prior to the injections and on the day following the last injection

TABLE 11

EFFECTS OF KAOLIN EXTRACTS ELUTED AT DIFFERENT *p*HS ON ERYTHROPOIESIS IN NORMAL RATS (Means ± Standard Errors)

Group	Treatment	Hematocrits (percentages)			Reticulocytes (percentages)			Body wts (gm)	
		Before	After	P	Before	After	P	Before	After
I	Saline control	47 5 ± 0 6	48 5 ± 0 8	NS	3 2 ± 0 9	3 4 ± 0 9	NS	185 ± 4	187 ± 2
II	*p*H 8 Eluate	47 5 ± 2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
III	*p*H 9 Eluate	46 9 ± 1							
IV	*p*H 10 Eluate	46 9 ± 1							
V	1 M NH_4OH Eluate	45 7 ± 1							
VI	2 M NH_4OH Eluate	46 9 ± 0							

It may be seen from TABLE 11 that the most active fractions were eluted at *p*H 8 0 and with 1 M NH_4OH Metachromasia was tested for by adding 0 05 ml of an 0 025 per cent solution of toluidine blue O dissolved in 1 M KH_2PO_4 to 0 2 ml of the 5 eluates Only the 1 M NH_4OH eluate produced a strong metachromatic reaction No metachromasia was elicited by the original urine or by the fractions eluted with the less basic buffers The failure of the original urine and the *p*H 8 eluate to exhibit metachromasia may have been due to the presence of metachromasia inhibitors Indeed, we have found that heparin does not display its usual pink metachromasia when added to anemic human urine

The nondialyzable solids content of the 1 M NH_4OH eluate was determined by dialysis, against distilled water, of 4-ml aliquots of the dialysand for 36 hours and evaporation to dryness Calculations indicated that a 39 per cent yield with approximately a two hundred and thirtyfold purification of the active factor had been obtained in this eluate The material was found to possess an absorption peak at 280 mμ with the DU spectrophotometer and to migrate very slowly in both the analytical centrifuge or on paper strips in an electrophoretic apparatus Applying the anthrone reaction, the 1 M NH_4OH eluate was found to contain approximately 25 per cent carbohydrate None of these findings is inconsistent with the contention that urinary ESF is a mucoprotein or an associated substance

TABLE 10
EFFECTS OF KAOLIN ELUATES CORRESPONDING TO 4 ADSORPTION pHs ON ERYTHROPOIESIS IN NORMAL RATS (Means ± Standard Errors)

Eluates injected	Hct (percentages)	Retics (percentages)
Adsorbed at pH 6 2		
Initial	48 7	2 8 ± 0 1
Final	49 1	5 8 ± 0 4 ($p < 0\ 01$)
Adsorbed at pH 5 2		
Initial	49 0	3 0 ± 0 5
Final	48 1	6 6 ± 0 4 ($p < 0\ 01$)
Adsorbed at pH 4 8		
Initial	47 7	2 5 ± 0 6
Final	49 2	6 7 ± 0 3 ($p < 0\ 01$)
Adsorbed at pH 4 3		
Initial	48 3	2 6 ± 0 6
Final	47 0	6 2 ± 0 5 ($p < 0\ 01$)
Buffer control		
Initial	49 6	2 5 ± 0 3
Final	43 8	2 4 ± 0 2

Third Kaolin Experiment

From the previous experiment, it was determined that pH 4 8 was as good as, if not better than, the other pHs for adsorption of the urinary factor the purpose of the third kaolin run to determine the best elution pH. adsorbed at higher pH solutions (gradient elution).

Method Three hundred ml of the same batch of erythropoietically active urine employed in the second kaolin experiment (TABLE 9) was used as the starting material Four and one-half gm of kaolin was added to the urine and stirred magnetically, 1 N HCl was added until a pH of 4 8 was attained The suspension was equilibrated for 24 hours at 5° C. The kaolin was equilibrated with a second

0 9 per cent saline again for 48 hours All eluates were approximately neutral, reading about *p*H 6 8

Six groups of adult female rats weighing 170 to 240 gm and averaging 185 to 195 gm per group were used for the assay Four rats comprised each group Each rat of the 6 groups received 3-ml subcutaneous injections of the following materials daily for 5 days Group I, sterile 0 9 per cent saline, Group II, *p*H 8 eluate in 0 9 per cent saline, Group III, *p*H 9 eluate in 0 9 per cent saline, Group IV, *p*H 10 eluate in 0 9 per cent saline, Group V, 1 M NH_4OH eluate, neutralized, in 0 9 per cent saline, Group VI, 2 M NH_4OH eluate, neutralized, in 0 9 per cent saline Reticulocyte counts and hematocrit determinations were made prior to the injections and on the day following the last injection

TABLE 11

EFFECTS OF KAOLIN EXTRACTS ELUTED AT DIFFERENT *p*HS ON ERYTHROPOIESIS IN NORMAL RATS (Means ± Standard Errors)

Group	Treatment	Hematocrits (percentages)			Reticulocytes (percentages)			Body wts. (gm)	
		Before	After	P	Before	After	P	Before	After
I									
II									1
III									0
IV									3
V									
VI									

It may be seen from TABLE 11 that the most active fractions were eluted at *p*H 8 0 and with 1 M NH_4OH Metachromasia was tested for by adding 0 05 ml of an 0 025 per cent solution of toluidine blue O dissolved in 1 M KH_2PO_4 to 0 2 ml of the 5 eluates Only the 1 M NH_4OH eluate produced a strong metachromatic reaction No metachromasia was elicited by the original urine or by the fractions eluted with the less basic buffers The failure of the original urine and the *p*H 8 eluate to exhibit metachromasia may have been due to the presence of metachromasia inhibitors Indeed, we have found that heparin does not display its usual pink metachromasia when added to anemic human urine

The nondialyzable solids content of the 1 M NH_4OH eluate was determined by dialysis, against distilled water, of 4-ml aliquots of the dialysand for 36 hours and evaporation to dryness Calculations indicated that a 39 per cent yield with approximately a two hundred and thirtyfold purification of the active factor had been obtained in this eluate The material was found to possess an absorption peak at 280 mμ with the DU spectrophotometer and to migrate very slowly in both the analytical centrifuge or on paper strips in

[illegible]

is a mucoprotein or an associated substance

The following experiment demonstrates that the polypeptide portion of this urinary mucoprotein is essential for its erythropoietic stimulating activity. A sample of urine from a Cooley's anemia patient with established activity was incubated with crystalline pepsin, trypsin, or chymotrypsin in the amount of 1 mg per ml of urine for 2½ hours at 37° C. The pepsin digest and a control without added enzyme were incubated at *p*H 1.9 Similarly, the trypsin digest and control were maintained at *p*H 8 6 The chymotrypsin treatment was conducted at *p*H 7.6 Three-ml. subcutaneous injections of each of the 5 solutions were administered daily for 5 days to groups of 4 intact female rats, and determinations were made on the sixth day

TABLE 12
EFFECTS OF PROTEOLYTIC ENZYME DIGESTION ON ERYTHROPOIETIC ACTION OF COOLEY'S URINE

Treatment	Retics, change in percentage	Hct, change in percentage
Trypsin	+0.8	−1.3
Trypsin control	+4.4*	+1.1
Pepsin	−0.1	+0.5
Pepsin control	+1.0	−1.2
Chymotrypsin	+0.9	−1.0

* Increase greater than control, $p < 0.01$

TABLE 12 indicates that only the trypsin control produced a significant reticulocytosis In the other cases, no hematocrit or reticulocyte changes were significant when compared to the controls

Since the low *p*H of 1 9 was sufficient to inactivate the factor, it cannot be concluded that the pepsin specifically destroys its activity. However, since the factor remains stable at a *p*H of 8 6, it can be inferred that the activity was abolished by trypsin Although no control was run at *p*H 7.6 (a *p*H at which the factor is probably stable), it appears that chymotrypsin also inactivates the factor In agreement with the results of Van Dyke and Garcia[22] working with urinary erythropoietin and with those of Slaunwhite *et al* [39] and Borsook[40] dealing with the plasma factor, these results indicate that the essential part of the erythropoietin molecule is a polypeptide. The demonstration by Van Dyke[41] that periodic acid also inactivates the factor does not

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We have noted on occasion that erythromacrocytosis is produced by active urines and by the eluates derived from the kaolin adsorptions This phenomenon has also been reported by Rambach *et al* [12] who found a shift of the Price-Jones curve to the right with acidified boiled filtrates of anemic plasma

We are at a loss to explain why microcytosis occurs on some occasions and why on others, especially with urinary material, macrocytosis occurs. In this connection, it would be of interest to test the combined effects of the urinary ESF and the plasma microcytosis factor of Linman and Bethell.

Studies of Blood Formation in Isolated Perfused Hind Limbs of Rats

Practically no information is available on bone marrow function in isolated perfused preparations. The classic experiments of Drinker *et al.*,[43] dealing with the perfusion of hind limbs of dogs, were concerned primarily with the cellular alterations in the perfusing fluid, and relatively little attention was given to the alterations in the marrow.

The purpose of this section of the paper is twofold: (1) to present a technique for the perfusion of hind limbs of rats that permits assessment of activity within the contained bone marrow and (2) to describe the effects of normal blood and blood containing the ESF on the marrow elements in isolated perfused hind limbs from normal and hypophysectomized rats. Abstracts covering several phases of this work have already appeared.[44, 45]

Technique for Perfusion of Rat Hind Limbs

The perfusion technique utilized a standard Dale-Schuster pump and an apparatus modified to recirculate a relatively small volume of blood (minimum of 15 ml.). Special glass parts were required for this purpose (FIGURE 4). A pulsatile flow was obtained by the addition of an air bell to the arterial side of the perfusion apparatus (FIGURE 4). A conventional-type mercurial manometer permitted the recording of perfusion pressures throughout the course of the experiments. The entire unit, with the exception of the manometer, the oxygenating chamber, and the limb to be perfused were submerged in a water bath maintained at 37° C. The perfused limb was kept in a moist chamber at 29 to 30° C (FIGURE 5).

In all experiments, the rats (Holzman strain, approximately 300 gm. in weight) used for the perfusion studies were first anesthetized with 40 mg. of sodium pentobarbital per kg. body weight. This was administered intraperitoneally as a 1.0 per cent solution. If necessary, ether was supplied to establish the desired depth of anesthesia. Following deep sedation, 5.0 mg. of heparin in 0.5 ml. of saline was injected into the femoral vein of the right leg. The rat was fastened to a cork board for cannulation of the vessels of the left leg. The abdomen was opened along the median ventral line. Following exposure of the major vessels (abdominal aorta, inferior vena cava, and iliac veins and arteries), the blood vessels to and from the right leg were tied off caudal to the bifurcation. Subsequently, the arterial cannula was inserted into the abdominal aorta just above the bifurcation and forced into the left iliac artery, at which point it was tied. The venous cannula was inserted into the inferior vena cava and tied just anterior to the bifurcation. The approximate time for the entire procedure was 5 min. However, blood was perfused through the left leg at sporadic intervals following insertion of

the arterial cannula, thus reducing the time of anoxia to considerably less than 5 min

Blood for these experiments was obtained from unanesthetized rats To prevent coagulation, each syringe contained 0 8 mg. of an 0 2 per cent solution of heparin A total of 45 to 50 ml was available for each experiment. This large volume was necessary in order to supply aliquots required for the



FIGURE 3 Schematic diagram of perfusion apparatus Reproduced by permission from *The American Journal of Physiology* [14]

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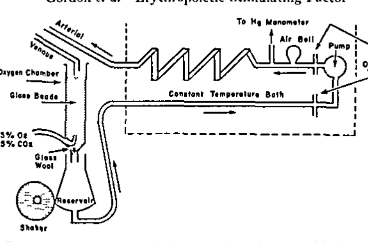


FIGURE 4 Specially constructed glass parts for perfusion assembly (Kuna *et al*, unpublished)

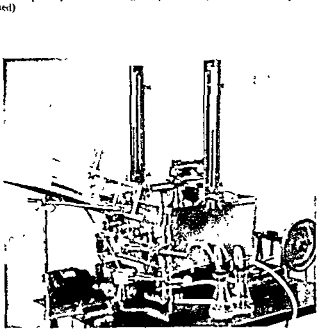


FIGURE 5 Photograph of entire perfusion system. Note moist chamber in which perfused limb is enclosed. Reproduced by permission from *The American Journal of Physiology*.[11]

Evidence for the maintenance of the functional integrity of the limb with its included marrow was made evident from

(1) Prompt vasoconstrictor response of the limb upon addition of epinephrine (1 μg per 50 ml of perfusate)

(2) Absence of demonstrable rigor and edema in the limb

(3) Constant utilization of glucose and oxygen

(4) Maintenance of normal biochemistry of the perfusate Determina-

occurred an increase in the concentration of inorganic phosphate which could not be prevented by the addition of adenylic acid, malic acid, Versene, inosine, calcium chloride, calcium gluconate, cortisone, ATP, or a mixture of creatine, hexosephosphate, and adenylic acid

(5) Accumulation of mitoses in both the erythroid and myeloid series following addition of colchicine to the perfusate (0.2 to 1.0 μg. per ml of perfusate)

(6) Incorporation of added C^{14}-glycine into radioactive hemin isolated from the red cells of the perfusate (15 μc of C^{14}-glycine added per 50 ml of perfusate)

Effects of Blood Obtained from Hypoxic Rats upon Erythropoiesis in Isolated Perfused Hind Limbs of Rats

Having determined that the limb operates with good efficiency during the 4-hour perfusion period, the next phase of the study was concerned with determining whether blood containing erythropoietic stimulating activity would enhance erythropoiesis in these isolated perfused preparations Whole blood obtained from the following types of animals was employed. Group 1, normal rats, Group 2, rats exposed 2 hours daily to lowered barometric pressures of 520 mm Hg, lowered gradually to 430 mm. Hg over a period of 15 or more days, Group 3, rats subjected to 5-ml. blood withdrawal on 2 to 3 occasions over a 10-day period, Group 4, rats subjected to a single hemor-

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percentage change

) from those in the

control contralateral nonperfused limbs Three to 5 experiments were performed for each of the 5 groups

It will be observed that perfusion of normal rat limbs with blood from normal rats (Group 1) did not alter significantly the numbers of erythroblasts compared to those found in the control nonperfused limbs. The slight

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hydrazine-treated (Group 5) rats. The most marked increases in the numbers of erythroblasts (approximately 70 to 90 per cent) were evoked with blood obtained from repeatedly bled and from phenylhydrazine-treated animals. Here, significantly increased numbers of mitotic figures were evident among the members of the erythroblast series.

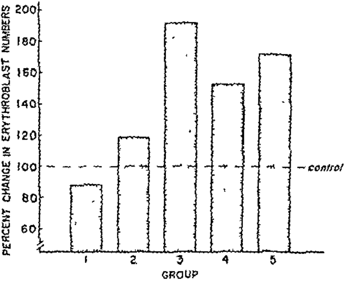


FIGURE 6. Erythropoietic effects of perfusing hind limbs of normal rats with blood from normal and hypoxic rats (Kuna *et al.*, unpublished).

It may be concluded from this study that the ESF present in the blood of animals subjected to hypoxic or anemic hypoxia exerts its stimulatory influence directly upon the blood-forming tissues and does not require the mediation of other organ systems.

On theoretical grounds, it might be expected that if the regulation of erythropoiesis is mediated by humoral mechanisms, the circulating ESF should be present in the blood of normal animals as well as in that of animals subjected to hypoxic stimuli. The inability to detect the undoubtedly smaller quantities of ESF in *normal* blood might be due to the utilization for perfusion, of *normal rat bone marrow* in which the rate of erythropoiesis is already geared at a high level.

It was felt that the use of limbs from hypophysectomized rats, under conditions of perfusion, would reflect more sensitively any ESF that might be present in normal blood. We[10] and others[17] have already indicated the greater erythrogenic response of hypophysectomized rats to material containing the ESF.

Two representative experiments involving perfusion of limbs obtained from hypophysectomized rats at 2 to 4 weeks following the operation and

Evidence for the maintenance of the functional integrity of the limb with its included marrow was made evident from:

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(2) Absence of demonstrable rigor and edema in the limb.

(3) Constant utilization of glucose and oxygen.

(4) Maintenance of normal biochemistry of the perfusate. Determina-

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erythroblasts within the femoral marrows of isolated perfused hind limb from normal rats. The values are expressed as average percentage chang of the erythroblast count (numbers/cu. mm of marrow) from those in th control contralateral nonperfused limbs Three to 5 experiments wer performed for each of the 5 groups.

It will be observed that perfusion of normal rat limbs with blood fror normal rats (Group 1) did not alter significantly the numbers of erythroblast compared to those found in the control nonperfused limbs The sligh

be due to the greater sensitivity to erythropoietic stimuli of the depressed erythrogenic marrow characteristic of the hypophysectomized animal. Experiments are being conducted to establish the nature of this stimulatory influence. Hypophysectomized rat limbs will be perfused with blood from hypophysectomized rats. The effects of hormones added to the perfusate, singly and in combination, will be studied. With this approach, it will be possible to determine whether the erythropoietic stimulating effects of normal blood, as observed in perfused hypophysectomized rat limbs, are due to (1) erythropoietic hormonal factors, such as the growth and thyroid principles, present in normal blood but not in the blood of hypophysectomized

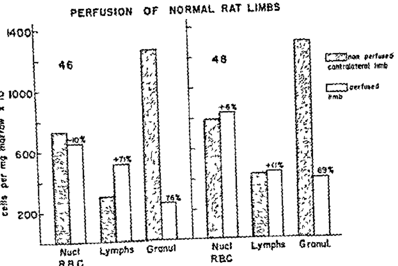


FIGURE 8. Marrow cellular numbers in normal rat limbs perfused with blood from normal rats (Dornfest *et al.*, unpublished).

animals, (2) nonspecific nutrients such as protein to which the erythropoietic processes in hypophysectomized rats respond sensitively,[24] and (3) the specific ESF that should be present in the blood of normal rats (although in smaller quantities), as well as in the blood of animals suffering from lack of oxygen.

Summary

Reasons are discussed for applying caution in relying too heavily on single parameters in the assessment of erythropoietic activity.

Strong erythropoietic stimulating activity is present in boiled filtrates of plasma from subjects with Cooley's and hypoplastic anemias. Transfusion of these patients with compatible blood or packed red cells to normal hemoglobin levels results in the rapid disappearance of the ESF from the plasma

from 2 intact normal rats are indicated in FIGURES 7 and 8, respectively. In all 4 cases, whole blood obtained from intact normal rats of the same strain was used as the perfusion medium. The following facts emerge from a consideration of these figures.

(1) The numbers of nucleated red cells are considerably less in the femoral marrows of hypophysectomized rats 2 to 3 weeks after the operation than in intact rats, lymphocyte numbers are significantly greater.

(2) Perfusion of limbs of hypophysectomized rats with blood of intact rats results in large increases in the numbers of nucleated red cells within the

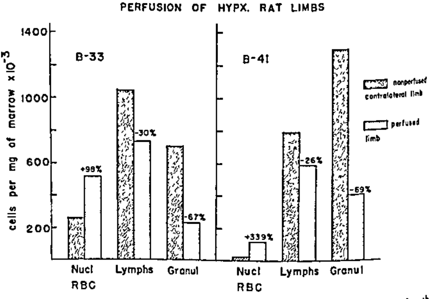


FIGURE 7 Marrow cellular numbers in hypophysectomized rat limbs perfused with blood from normal rats (Dornfest, Gordon, and Siegel, unpublished)

femoral marrows, at the same time, the numbers of marrow lymphocytes are decreased A considerable lowering in the numbers of granulocytes (mostly of the segmented variety) also occurs

(3) Perfusion of limbs from intact rats with blood from intact rats exerts no significant influence on the numbers of nucleated red cells within the marrow. Lymphocyte numbers are either not altered or exhibit a rise The numbers of granulocytes decline in much the same way as they do in the perfused limbs of hypophysectomized rats Dornfest *et al*[46] have found recently that this is due largely to a release of granulocytes from the marrow into the per

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Unconcentrated urine from these patients contains considerable quantities of the ESF. However, activity is not always present, and this may be the result of normal or pathological changes in kidney threshold for the factor.

The urinary erythropoietic factor may be adsorbed on kaolin in acid pH and eluted at several pHs in the alkaline range. The greatest activity is obtained in the 1 M NH_4OH eluate, in which a purification of 230X has been achieved. The addition of toluidine blue to this eluate, results in strong metachromasia. UV absorption spectra studies indicate that the biologically active fractions absorb considerable light at 280 mμ. These properties support the contention that the ESF is a mucoprotein or an associated substance. Biological activity is destroyed by proteolytic enzymes, indicating the importance of the polypeptide portion of the urinary mucoprotein.

Blood from [illegible] treated rats [illegible]

blood is shown to contain a factor(s) capable of stimulating erythropoiesis [illegible]

[illegible] ction

[illegible] n the

treatment of certain blood dyscrasias in man.

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CURRENT STUDIES ON THE ROLE OF ERYTHROPOIETIN ON ERYTHROPOIESIS

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Introduction

The existence of a humoral erythropoietic stimulating factor that controls red cell production has been suspected for many years Carnot and Déflandre[1] postulated in 1906 that this might be true In 1950 Reissmann[2] published convincing experimental evidence, based on the use of parabiotic rats, of a circulating erythropoietic factor induced by placing one member of the pair in a hypoxic atmosphere In 1952 an excellent review concerning the fundamental stimulus for erythropoiesis was written by Grant and Root[3] A blood-borne substance capable of stimulating erythropoiesis was strongly suggested Bonsdorff and Jalavisto[4] referred to this substance as erythropoietin Subsequently many laboratories, using several different methods of study, have taken up and expanded this concept Thus plasma, or a "deproteinized" extract of plasma obtained from animals rendered anemic by bleeding,[5-11] was shown to have the ability to stimulate increased erythropoiesis in recipient animals Similar material from animals with phenylhydrazine-induced hemolytic anemia[12-16] possessed the same, although somewhat more potent, properties Plasma from animals kept in a low oxygen atmosphere has exerted significant erythropoietic activity[17-19] Also, there is evidence that a substance may be present in the milk of anoxic rats and mice that produces an increased hemoglobin content of the nursing young[20] As studies with experimental animals have progressed, increasing interest has been centered on the significance of these findings in patients with various types of anemia and polycythemia This phase, the study of human patients, has been aided tremendously by the demonstration[13, 21, 22] that boiled extracts of human plasma and urine retain their erythropoietic-stimulating properties These boiled extracts of human plasma and urine could be assayed using small amounts of plasma and urine in other animal species without detectable reactions due to foreign protein

It is the purpose of this communication to report some of our findings with experimental animals and human patients The areas of the problem to be discussed are

Site of production of erythropoietin (1) relationship of the kidney, (2) relationship of the reticuloendothelial system; and (3) relationship of the bone marrow

Relation of the liver to erythropoietin (1) direct evidence, and (2) indirect evidence

The effect of various hormones and other known erythropoietic substances on erythropoietin levels

Erythropoietic-stimulating effect of cobalt

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TABLE I
HYPOPHYSECTOMIZED RATS (RECIPIENTS) RECEIVING PLASMA FROM NORMAL SPRAGUE-DAWLEY RATS (DONORS) THAT WERE NEPHRECTOMIZED AND SPLENECTOMIZED AND KEPT IN 10 PER CENT OXYGEN FOR VARYING PERIODS
Erythropoiesis in Recipient as Adjudged by Fe^{59} Uptake

Treatment of donor rats prior to placing in 10 per cent O_2 atmosphere	Interval at 10 per cent O_2 (hours)	No recipient rats	24-hour Fe^{59} uptake
Unilateral nephrectomy	4	5	13 9 ± 4 13*
	8	5	31 2 ± 5 84
	24	6	40 0 ± 5 66
Bilateral nephrectomy	4	6	17 9 ± 3 06
	8	5	13 2 ± 4 23
	24	6	37 9 ± 2 53
Sham nephrectomy	4	6	21 8 ± 4 84
	8	5	29 1 ± 6 41
	24	6	35 4 ± 7 70
Splenectosny	4	4	25 1 ± 1 70
	8	5	33 3 ± 6 37
Splenectomy-unilateral nephrectomy	4	6	21 8 ± 5 37
	8	5	37 8 ± 11 45
Splenectomy-bilateral nephrectomy	4	4	13 4 ± 5 36
	8	6	17 6 ± 1 91
Sham splenectomy-nephrectomy	4	6	22 4 ± 5 12
	8	6	35 1 ± 4 78
Normal rat plasma	–	5	7 6 ± 2 69
Untreated	–	6	6 7 ± 4 0

* Plus or minus S D
Assays in all groups were carried out at the same time and under similar circumstances
In no instance did similar treated rats at normal atmosphere show a significant elevation of erythropoietin titers

the chamber at all times At the end of the respective intervals rats were withdrawn from the chamber individually and promptly exsanguinated from the dorsal aorta Plasma obtained was frozen and stored until used

For our indirect assay, recipient animals were 3-month-old Sprague-Dawley rats used approximately 15 to 20 days after hypophysectomy [27,28] They received 2 cc of donor rat plasma to be tested I V via the jugular vein on 2 successive days On day 3 approximately 1 μc Fe^{59} was given I V via femoral vein and on day 4 a 24-hour Fe^{59} uptake was done The results for each group of animals were expressed as the average 24-hour Fe^{59} uptakes for each particular group FIGURES 1 and 2 show averaged Fe^{59} uptakes of hypophysectomized recipient rats that received plasma from donor rats subjected to various combinations of experimental conditions Each figure represents a compilation of averages of 24-hour Fe^{59} uptakes of 3 separate experiments representing in each experiment 25 experimental conditions The combined experiments represented in FIGURES 1 and 2 were not analyzed together because the data were complicated by the fact that, in general, there

Comparison of erythropoietic activity of boiled *vs.* unboiled plasma.
The chemistry of erythropoietin.
Erythropoietic activity of plasma and urine of human subjects

Site of Production of Erythropoietin

Relationship of the kidney Gordon *et al.*[23] attempted unsuccessfully to identify the site of production of erythropoietin by assaying acidified boiled filtrates prepared of plasma, packed blood cells, and various organs Only the filtrates of plasma stimulated erythropoiesis in recipient rats Jacobson *et al* [24] reported that the removal of the pituitary, thyroid, spleen, adrenal, gonads, stomach, pancreas, intestine, and a major part of the liver does not impair the ability to produce erythropoietin under the stimulus of bleeding, cobalt, or hypoxia anoxia [25] However, after bilateral nephrectomy neither rats nor rabbits had the capacity to respond to bleeding or cobalt or hypoxic anoxia and therefore Jacobson *et al.*[24] postulated that the kidney is the major source for erythropoietin or an inactive precursor that is activated by some other tissue Erslev[26] reported that observations on uremic rabbits do not support Jacobson's concept because serum rendered both anemic and uremic showed a low level of erythropoietin whether the uremia was induced by bilateral nephrectomy or by bilateral ureter ligation. Moreover, the bone marrow of nephrectomized rabbits continued to be active despite the complete absence of the kidney Mirand and Prentice[18] showed the presence of plasma erythropoietin in hypoxic anoxic rats with or without kidney(s) and/or spleen (TABLE 1) Further observations are presented on the production or lack of production of erythropoietin in rats with intact kidneys after bilateral nephrectomy or after bilateral ureter ligation when subjected to bleeding, cobalt administration, and/or hypoxia, and on the ability of hypophysectomized rats to produce erythropoietin under various experimental conditions

The procedures used in this study are as follows Sprague-Dawley rats were placed in chambers constructed of Plexiglas. Into each chamber was directed a mixture of nitrogen and oxygen, the proportion of which, along with ambient pressure, determined the simulated altitude. Flow of nitrogen and oxygen was regulated by a rotameter type of flowmeter and remained

Treatments are ranked in FIGURES 1 and 2 from 1 to 25 Differences between the various pairs are compared with the standard deviation multiplied by appropriate constants from tables of ranges in the above-mentioned article [29] Any two treatments that exceed the appropriate tabulated value (when multiplied by the standard deviation) are declared to be significant at the 5 per cent level of significance

Direct assays were carried out on Sprague-Dawley rats, 400 to 500 gm The treatment and Fe^{59} assay were performed on the same rat (TABLE 2) A ngle hemorrhage, cobalt dose, or anemic plasma was given after the removal f kidneys, and Fe^{59} was injected I V via the jugular vein at this time The ats were sacrificed later for a 24-hour Fe^{59} uptake

TABLE 2
DIRECT ASSAY

Treatment	Rats with			
	Presence of kidney		Absence of kidney	
Groups	N A	Low O_2	N A	Low O_2
Untreated	26.6* (±3.9)†	35.2 (±4.2)	23.6 (±4.5)	43.1 (±2.4)
Bled (6 cc)	41.4 (±5.0)	40.0 (±3.0)	23.8 (±4.4)	32.1 (±7.5)
Cobalt (10 mg)	28.0 (±5.0)	18.2 (±5.0)	23.7 (±4.8)	19.3 (±1.2)
Cobalt (15 mg)	31.9 (±3.6)	18.9 (±4.3)	20.3 (±2.2)	12.5 (±1.2)
Anemic rat plasma (LPI) (2 cc)	29.5 (±1.5)	38.5 (±2.2)	19.9 (±6.0)	30.2 (±1.2)

Key N A , Normal atmosphere, Low O_2 10 per cent oxygen level for 24 hours, EPF, ythropoietic factor
* Average 24-hr Fe^{59} uptakes
† Standard deviation

Published data from this laboratory[19] have shown that when normal rats re exposed to an atmosphere of approximately 10 per cent O_2 for 4, 8, 24, 48, nd 120 hours, a time-concentration relationship of plasma erythropoietin EPF) in hypoxic anoxic rats can be seen The concentration is approximately 5 times the control level at 4 hours, at 8 hours the titer reaches 10 imes control level and remains approximately the same at 24 hours By 48 nd 120 hours the titer has dropped back to control levels

Using this time-concentration relationship of plasma erythropoietin in ypoxic anoxic rats as a basis, we set out to determine whether this relationhip could be altered by nephrectomy and or splenectomy in order to reveal he site of production of hypoxic erythropoietin TABLE 1 shows that the ime-concentration relationship of plasma EPF in unilateral or bilateral ephrectomized animals subjected to hypoxic anoxia is doubled at 4 hours, doubled or quadrupled at 8 hours, and is 5 to 6 times greater at 24 hours han the titers at control levels When splenectomy was combined with ach of the first 3 procedures, essentially the same results were obtained

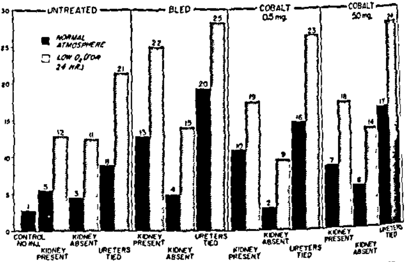


FIGURE 1 Relationship of the kidney to erythropoietin average Fe^{59} uptakes of Hypx rats receiving donor rat plasmas

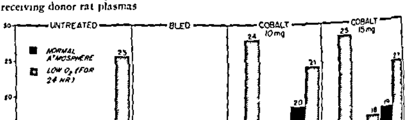


FIGURE 2 Relationship of the kidney to erythropoietin average Fe^{59} uptakes of Hypx rats receiving donor rat plasmas

were unequal numbers of observations in the subclassifications formed by various combinations of experimental conditions and by different days of repetition of the experiment. The average variation in the material, as represented by the standard deviation, was estimated to be 0.32 per cent. It was against this inherent variation that the significance of the treatment effects were judged. A procedure for determining whether any 2 treatment effects differ significantly from each other is the Multiple Range Test.[22]

kidneys (compare bars 6 and 8 with 5) FIGURE 1 illustrates a similar trend with lower doses of cobalt The combination of cobalt plus hypoxic anoxia can increase the erythropoietin titer in the presence of the kidney (compare bars 24 and 25 with 11) and in the absence of the kidney (compare bars 17 and 18 with 9), but not necessarily so after bilateral ureter ligation (compare bars 21 and 22 with 23) Severe anoxia resulting from bleeding plus hypoxic anoxia in rats with kidney absent (compare bar 4 with 9) or after ureter ligation (compare bar 15 with 23) can prevent the appearance of elevated erythropoietin titers Other data observed in our laboratory further show that a severe hypoxic anoxia per se (as also reported by Stohlman and Brecher[17]) or combinations of severe bleeding plus hypoxic anoxia or large doses of cobalt plus hypoxic anoxia can decrease the titer of plasma erythropoietin.

In TABLE 2 "direct assay" refers to the treatment and assay performed in the same rat, in contrast to an "indirect assay" (FIGURES 1 and 2) in which the treatment was performed on a donor rat and the plasma was assayed in recipient hypophysectomized rats It can be observed further in TABLE 2 that a hypoxic anoxic stimulant can produce erythropoietin in absence of the kidney (compare average 24-hour, Fe^{59} uptake values 23 6 and 43 1 in untreated group) Also, rats in a normal atmosphere subjected to bleeding cannot elicit erythropoietin in the absence of the kidney (compare values 41 4 with 23 8) Rats with or without kidneys in normal atmosphere and receiving cobalt did not, or did not markedly, demonstrate an increase in erythropoietin titers, moreover, the combination of cobalt with hypoxic anoxia demonstrated a decreased titer of erythropoietin regardless of whether the kidney was present It is interesting that a single injection of anemic plasma containing erythropoietin did not stimulate erythropoiesis

The data in FIGURE 3 show that donor plasma of hypophysectomized rats or hypophysectomized rats with the removal of additional organs can demonstrate an increased erythropoietin titer following exposure to hypoxic anoxia at various intervals of 4, 24, and 48 hours Likewise, hypophysectomized rats can demonstrate an increase of erythropoietin after the single removal of 4 cc of blood The combination of a single exposure to hypoxic anoxia and a single dose of cobalt (1 5 mg) does not produce an additive effect on erythropoietin titers In fact, cobalt plus hypoxic anoxia reduces the amount of erythropoietin as seen in the 4-, 24- and 48-hour hypoxic groups Note that binephrectomized and hypophysectomized rats can respond to hypoxic anoxia However, the erythropoietin is reduced as compared with hypophysectomized controls

These studies demonstrate that the kidney plays an important role in the production of erythropoietin The studies of Jacobson *et al*[14] showing that the kidneys, when removed, fail to produce erythropoietin under the erythropoietic stimulus of bleeding or cobalt administration have been confirmed by our studies using indirect and or direct assay methods The fact that cobalt plus hypoxia depresses erythropoiesis in rats in our direct assay data (TABLE 2) might mean that cobalt at these concentrations "poisons" hemoglobin synthesis, but does not interfere with erythropoietin production

Nephrectomized and/or splenectomized rats exposed to normal atmosphere did not show any elevation of erythropoietin. Their 24-hour Fe^{59} uptakes were comparable to values for hypophysectomized rats receiving normal plasma From such data it was concluded that hypoxic EPF production was not dependent on the presence of the kidney and/or spleen

Further extensive experiments investigating the relationship of the kidney to erythropoietin under normal and hypoxic atmospheres were performed FIGURE 1 shows a compilation of averages of 24-hour Fe^{59} uptakes of 3 separate experiments representing, in each experiment, 25 experimental conditions For each separate experiment the assay procedures on 25 experimental conditions were carried out simultaneously.

In FIGURE 1 the bottom line represents the status of the kidney of dono rats and the line above represents the treatment they received. The dono rat plasma was assayed in hypophysectomized rats by use of the 24-hou Fe^{59} uptake as shown by the height of each bar. A difference of 2.9 per cen between any 2 averaged 24-hour Fe^{59} uptakes is significant at 5 per cent, a determined by the Multiple Range Test From FIGURE 1 the following state

after bilateral ureter ligation, as well as in the presence of the kidney. Eryth ropoietin elicited by bleeding is contingent upon the kidney, since detectab elevations in erythropoietin titers can be observed in response to a sing hemorrhage in the presence of the kidney (compare bar 13 with 5) or aft bilateral ureter ligation (compare bar 20 and 8), but *not* in the absence of tl kidneys (compare bar 4 with 3) under normal atmosphere The combinatic of bleeding plus hypoxic anoxia can increase erythropoietin titers in tl presence of the kidney (compare bar 22 with 12) or after bilateral uret ligation (compare bar 25 with 21). Note that bleeding plus hypoxic anox does not demonstrate a significantly higher rise in erythropoietin in tl absence of the kidney (compare bar 15 with 11) That erythropoietin appea to pile up in the plasma due to the inability of the rat to excrete it is demo strated by higher levels of erythropoietin titers in rats with bilateral uret ligation subjected to normal or hypoxic atmospheres (compare bar 8 with 20 with 13, 16 with 10, 17 with 7, 21 with 12, 25 with 22, 23 with 19, ai 24 with 18).

FIGURE 2 shows a similar compilation of averages of 24-hour Fe^{59} uptak of 3 separate experiments representing, in each case, 25 experimental con tions These experiments differ from the previous ones in FIGURE 1 by usi a more severe single hemorrhage and a larger cobalt dose to donor rats

nd placed immediately, or eighteen hours later, in an atmosphere of 10 per cent O_2 for 4 to 24 hours From the data in FIGURE 4, it can be observed that donor plasma of rats so treated, when assayed in recipient hypophysectomized rats, still demonstrate EPF in their plasma However, it must be borne in mind that perhaps complete blockage of the RES was not achieved with the dose of thorotrast given

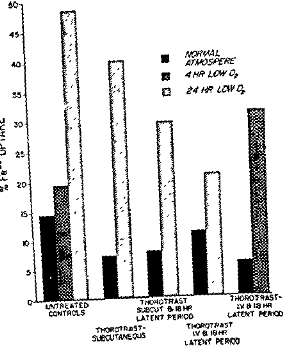


FIGURE 4 Effect of thorotrast on plasma erythropoietin

Relationship of the bone marrow Donor rats were exposed to whole body radiation with the following doses 200 r, 400 r, 600 r, 800 r, 1000 r, 1200 r, and 1400 r From the first to the seventh day after radiation the donor rats, after being exposed to a hypoxic anoxic stimulus for 24 hours, were sacrificed and their plasma assayed for erythropoietin In all groups, the radiated donor rats exposed to a hypoxic anoxic atmosphere for 24 hours demonstrated the presence of erythropoietin in recipient hypophysectomized rats as judged by a 24-hour Fe^{59} uptake This would support the hypothesis that the bone marrow is not the site for erythropoietin production

Relationship of the Liver to Erythropoietin

Direct evidence FIGURE 5 demonstrates the *in vitro* inactivation of erythropoietin by the liver By injecting 2 cc of phenylhydrazine-anemic plasma

The erythropoietin under these circumstances could very well accumulate in the plasma, and yet the bone marrow for some unknown reason remains refractory to the erythropoietin released by cobalt.[30] However, in the indirect assay where hypoxic cobalt plasma obtained from hypophysectomized rats was assayed in recipient hypophysectomized rats, we see a decrease in EPF titer (FIGURE 3) Further investigations on these points are necessary

These experiments using both direct and indirect assays further support our original concept that the kidney is not necessary for the production of erythropoietin under a hypoxic anoxic stimulus We have obtained plasma on occasion with little or no EPF, particularly from young donor rats exposed

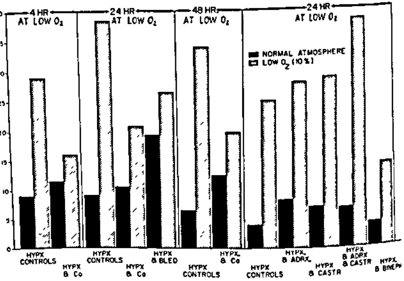


FIGURE 3 Average Fe^{59} uptakes of Hypx rats receiving donor Hypx rat plasmas

... Perhaps the fact that the ... maximal stim... However, th... e kidney wou...
... appear to rule out a unitarian concept for a source of erythropoietin. Ju... where hypoxic anoxic erythropoietin is produced remains unknown Th... [32, 33] bleeding ... n the plasm... [34] as a sour... of erythropoietin.[34]

Relationship of the reticuloendothelial system Thorotrast is known ... block the activity of the reticuloendothelial system (RES). Since this is ... general system in the body, we thought that blockage of the RES with thor... trast might diminish the production of erythropoietin. Therefore, don... rats were injected subcutaneously or intravenously with thorotrast (10 cc /k...

and placed immediately, or eighteen hours later, in an atmosphere of 10 per cent O_2 for 4 to 24 hours. From the data in FIGURE 4, it can be observed that donor plasma of rats so treated, when assayed in recipient hypophysectomized rats, still demonstrate EPF in their plasma. However, it must be borne in mind that perhaps complete blockage of the RES was not achieved with the dose of thorotrast given.

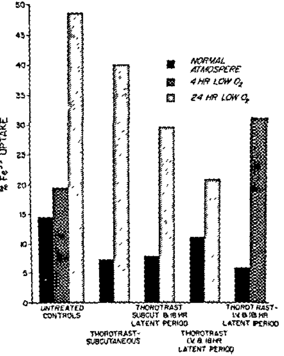


FIGURE 4. Effect of thorotrast on plasma erythropoietin.

Relationship of the bone marrow. Donor rats were exposed to whole body radiation with the following doses: 200 r, 400 r, 600 r, 800 r, 1000 r, 1200 r, and 1400 r. From the first to the seventh day after radiation the donor rats, after being exposed to a hypoxic anoxic stimulus for 24 hours, were sacrificed and their plasma assayed for erythropoietin. In all groups, the radiated donor rats exposed to a hypoxic anoxic atmosphere for 24 hours demonstrated the presence of erythropoietin in recipient hypophysectomized rats as judged by a 24-hour Fe^{59} uptake. This would support the hypothesis that the bone marrow is not the site for erythropoietin production.

Relationship of the Liver to Erythropoietin

Direct evidence. FIGURE 5 demonstrates the *in vivo* inactivation of erythropoietin by the liver. By injecting 2 cc. of phenylhydrazine-anemic plasma

from otherwise normal rats via the hepatic portal or jugular vein of recipient hypophysectomized rats, a clear-cut differential in 24-hour Fe^{59} uptakes can be seen

Recent preliminary data demonstrate the inactivation of anemic rat plasma when perfused through an isolated liver preparation for 3 hours [11]

Indirect evidence Additional indirect experimental evidence showing that endogenic hypoxic anoxic erythropoietin is inactivated by the liver is supported by the experiments of [illegible]
are placed in low ox[illegible]
48 hours or 5 days, [illegible]

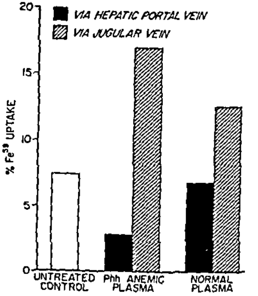


FIGURE 5 Hypx rats given phenylhydrazine anemic rat plasma via the hepatic portal or jugular vein

of their plasma in either recipient normal or hypophysectomized rats. However, when acute liver damage was induced by carbon tetrachloride and the rats were placed at the same simulated altitude as the normals for 48 hours, their plasma possessed erythropoietin

Jacobsen *et al* [16] have demonstrated [illegible]

[illegible] significant amounts of EPF can be stimulated in an animal with no anemia, provided hypoxic anoxia and liver damage are present in combination

It is not too surprising to find that EPF can be inactivated by the liver, for substantial data for the inactivation of such compounds as estrogens, androgens, and adrenal steroids are well documented

Effect of Various Hormones and Known Erythropoietic Substances on Erythropoietin Levels as Adjudged by Fe^{59} Uptake

A group of normal rats was primed with subcutaneous injections of "Depo"-Testosterone cyclopentylpropionate (single injection of 2 mg/kg) and cobalt chloride (3 mg/kg, daily), or bled until a hematocrit of 25 or below was obtained. After being primed or bled for 7 days, they were given 1 µc Fe^{59}, I.V. and subjected to hypoxic anoxia of a 10 per cent O_2 level for 24 or 48 hours. In all instances a 24-hour Fe^{59} uptake was taken.

TABLE 3
Fe^{59} UPTAKE IN NORMAL RATS RECEIVING FOLLOWING TREATMENT

Direct assay	N	O_2 level (10 per cent—24 hrs.)
Testosterone	32.6 (±7.6)	47.2 (±2.7)
Estrogen	17.7 (±5.6)	31.2 (±2.1)
Erythropoietin	55.7 (±3.8)	60.0 (±2.3)
$CoCl_2$	42.3 (±4.9)	40.6 (±4.6)
Bleeding	73.7 (±6.7)	69.5 (±5.8)
Controls	35.8 (±3.0)	40.9 (±5.9)

TABLE 4
Fe^{59} UPTAKE IN NORMAL RATS RECEIVING FOLLOWING TREATMENT

Direct assay	N	O_2 level (10 per cent—48 hrs.)
Testosterone	27.4 (±4.3)	49.4 (±6.1)
$CoCl_2$	34.9 (±3.3)	45.3 (±6.0)
Bleeding	60.5 (±2.3)	75.2 (±3.2)
Controls	30.2 (±2.8)	41.0 (±6.9)

It can be observed from TABLES 3 and 4 that bleeding or the injection of boiled phenylhydrazine-anemic rabbit plasma or cobalt (to a slight degree) caused an increase in Fe^{59} uptakes of rats exposed to normal or hypoxic atmosphere. However, hypoxic anoxia in combination with bleeding, erythropoietin, or cobalt did not demonstrate an additive effect on Fe^{59} values. Testosterone did not materially increase Fe^{59} value of rats in normal or hypoxic atmosphere. Estrogen inhibited the ability of rats in normal or hypoxic atmosphere to produce and/or respond to erythropoietin.

Erythropoietic-Stimulating Effect of Cobalt

The mechanism of the erythropoietic-stimulating effect of cobalt is still in question. Recent investigation of this problem by Goldwasser and his co-workers[36, 49] has stimulated further interest in it and has suggested the

possibility that cobalt exerts its stimulating effect on RBC production through increased output of EPF. The present study was designed to test this theory further by determining the amounts of EPF in the urine and plasma of animals receiving cobalt over a long period of time during the induction of their polycythemia.

Animals used were Sprague-Dawley rats in the age range of 3 months at the onset of the experiments. One and one-half mg of cobalt chloride was injected daily subcutaneously for a period of 10 weeks. At the end of each

TABLE 5
ERYTHROPOIETIC STIMULATING EFFECT OF COBALT

Determinations	Cobalt administered for the following weeks							
	3	4	5	6	7	8	9	10
Fe^{59} uptake								
Exper	30 6 ±5 1	37 8 ±2 4	35 8 ±3 6	44 1 ±2 9	37.0 ±2 8	35 5 ±1 1	31 8 ±4 5	41 2 ±4 1
Control	30 8 ±5 9	31 0 ±1 9	30 8 ±3 3	26 4 ±5 6	35 9 ±4 9	28 0 ±3 0	31 4 ±2 8	33 6 ±4 7
Hematocrit								
Exper	49 0	51 5	56 7	61 8	65 8	67 0	69 0	68 7
Control	46 5	47 5	47 0	47 5	43 7	46 3	47 2	45 0
Hemoglobin								
Exper	16 3	16 1	18 2	20 0	21 4	21 1	22 4	21 7
Control	14 1	14 8	16 0	15 9	14 1	15 7	16 3	15 5
Marrow erythroid percentage								
Exper	26 1	29 0	47 2	45 7	Not done		34 6	44 6
Control	24 3	29 2	23 6	20 8	Not done		26 1	<5 0
Reticulocytes								
Exper	1 1	2 9	2 1	1 9	3 2	2 4	1 6	1 4
Control	2 0	0 6	0 8	0 8	0 4	—	1 5	0 1
Plasma activity (Fe^{59} uptake)								
Exper	11 2	8 3	8 8	5 7	9 6	10 3	7 4	4 4
Control	9 1	8 6	4 3	8 9	7 7	9 9	11 1	9 5
Urine activity (Fe^{59} uptake)								
Exper	—	20 2	—	21 0	23 1	18 3	23 2	23 0
Control	27 7	19 5	24 0	17 7	21 0	24 8	13 8	20 7

week a part of the total group was sacrificed and a series of determinations carried out in these animals. These consisted of a Hb, hematocrit, bone ... ticulo- p each say of nimals d [11, 12] trated ll was to one frozen until used. For testing purposes it was given in 2-cc. amounts sub-

utaneously for 3 days to 14-day hypophysectomized rats On day 4, 1 μc 'e[59] was given I V and, on day 5, a 24 hour Fe[59] uptake was done At the ermination of the experiment, a total red cell volume was done on 10 animals, sing Fe[59]-labeled red cells [41]

Over the 10-week period a very pronounced polycythemia developed 'his was progressive and accompanied by the usual signs of increased red cell roduction, as shown in TABLES 5 and 6 However, during this entire interal neither the urine nor the plasma showed any increased erythropoietic-timulating activity

TABLE 6
BLOOD VOLUME DATA IN EXPERIMENTAL AND CONTROL ANIMALS AT END OF 10 WEEK INTERVAL OF COBALT ADMINISTRATION

Group TBV	Hct percentage	TRCV (cc)	Body wt (gm)	TRCV cc/100 gm BW
Experimental (cc)				
31 8	73 0	23 2	328 0	7 07
34 3	75 0	25 7	352 0	7 30
31 5	73 0	23 0	327 0	7 03
27 3	72 0	19 7	326 0	6 04
28 5	73 0	20 8	334 0	6 23
30 7 av	73 2 av	22 5 cc av	333 4 av	6 73 av
Control				
24 9	43 0	10 7	445 0	2 40
24 6	43 0	10 6	447 0	2 37
29 6	48 0	14 2	492 0	2 89
26 3	44 0	11 6	433 0	2 68
23 6	48 0	11 3	390 0	2 89
25 8 av	45 2 av	11 7 av	441 4 av	2 65 av

These data illustrate that a significant polycythemia can be produced in its by means of cobalt-chloride administration in the absence of any demontrable increased titer of erythropoietin in plasma or urine Since the assay iethods used to obtain this data were among the most sensitive to demontrate EPF activity in plasma or urine, these data appear to be of interest t will be noted that the dose of cobalt chloride used in this experiment was 5 mg/day This is a smaller dose than that used by Goldwasser *et al*,[30] who used a dose of approximately 10 mg/day or, roughly, 6 times our dose level Using a similar dose level, we were able to confirm increased erythropoietin levels in the plasma after a single injection However, such a dose is toxic to rats, and these animals are unable to tolerate it beyond a short period of time

Therefore the question arises as to whether the polycythemia that has developed with this dose of cobalt is due to increased outflow of EPF. In view of the negative results from the assay of the plasma and urine, one could

draw two conclusions: (1) that the polycythemia occurring subsequent to this dose of cobalt was not due to increased amounts of erythropoietin, or (2) that there was a minimal rise in erythropoietin levels undetectable by our assay methods, but adequate to produce polycythemia when present for a period of several weeks. We are not prepared to state which of these possibilities represents the true state of affairs. However, the latter statement implies extreme difficulty in interpreting the importance of plasma and urine EPF levels in various types of anemia and polycythemia because conclusions cannot be drawn from the levels of EPF found by assay

Comparison of Erythropoietic Activity of Boiled Versus Unboiled Plasma

There have been differences in opinion for several years concerning the relative erythropoietic potency of boiled versus unboiled plasma This is

TABLE 7
THE COMPARISON OF ERYTHROPOIETIC ACTIVITY OF BOILED VERSUS UNBOILED PLASMA AS ADJUDGED BY Fe^{59} UPTAKE IN RECIPIENT HYPOPHYSECTOMIZED RATS

Injected material from normal rat (2 cc × 2 da)	Route of injection	Unboiled		Boiled	
		Percentage Fe^{59} uptake	Percentage Hct	Percentage Fe^{59} uptake	Percentage Hct
Bled anemic rat plasma	I V	30 7	47 8	16 9	47 8
Phenylhydrazine anemic	I V	34 8	47 8	12 4	50 8
Hypoxia rat plasma	I V	39 5	46 0	13 6	47 3
Control	I V	13 4	45 4	11 7	49 7
Untreated control		Fe^{59} uptake—10 6		Hct 49 4	
Bled anemic rat plasma	I V	16 2	46 6	10 4	46 6
Phenylhydrazine anemic	I V	19 6	45 0	15 4	49 6
Hypoxia rat plasma	I V	10 7	45 7	11 6	46 3
Control	I V	8 0	46 8	6 8	45 3
Untreated control		Fe^{59} uptake—3 0		Hct 47 5	

still unresolved Certain groups[13] continue to be convinced that little or no
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pared the relative efficacy of various routes of plasma administration (subcutaneously, I.P , and I V) The latter was done to ascertain the feasibility

(1) Whole

phenylhydrazine ... nately equal

erythropoietic activity whether given I V, I P, or subcutaneously (boiled plasma extract was somewhat more active by the I V route in this experiment, however, in previous reported experiments, comparable activity was found when boiled extract was given by the I V and subcutaneous routes) (2) Boiled plasma lost a large amount of its erythropoietic-stimulating qualities as compared with unboiled material This was true in short-term experiments (4 days) using 24-hour Fe^{59} uptake as the method of assay, and also in long-term experiments (2 weeks) where Hb, Hct, and TRCV were used

TABLE 8
COMPARISON OF ERYTHROPOIETIC ACTIVITY OF BOILED VERSUS UNBOILED PLASMA AND THE RELATIVE EFFICACY OF VARIOUS ROUTES OF PLASMA ADMINISTRATION AS ADJUDGED BY Fe^{59} UPTAKES IN RECIPIENT HYPOPHYSECTOMIZED RATS

Injected material from normal rat (2 cc × 2 da)	Route of injection	Unboiled		Boiled	
		Percentage Fe^{59} uptake	Percentage Hct	Percentage Fe^{59} uptake	Percentage Hct
Bled anemic rat plasma	I V	22 9	48 0	15 3	45 5
Bled anemic rat plasma	I P	21 0	47 0	8 2	48 3
Bled anemic rat plasma	S C	20 6	43 8	2 5	53 4
Phenylhydrazine anemic	I V	32 9	47 0	28 5	47 4
Phenylhydrazine anemic	I P	34 6	50 0	18 5	45 8
Phenylhydrazine anemic	S C	36 9	48 2	16 2	45 8
Hypoxia rat plasma	I V	19 6	47 0	9 1	48 4
Hypoxia rat plasma	I P	9 9	47 8	12 5	48 2
Hypoxia rat plasma	S C	4 2	45 2	4 1	48 3
Control	I V	8 6	45 2	12 9	49 8
Control	I P	5 5	52 2	4 7	46 7
Control	S C	7 5	51 0	7 3	47 0
Untreated control		Fe^{59} uptake—2 8		Hct 49 8	

as the end point On this question we accordingly find that boiled material maintains some erythropoietic activity, but considerably less (one third to one half) than unboiled plasma These results are in agreement with those reported by Stohlman[12] who utilized short-term studies with Fe^{59} uptake as the method of assay It has recently been reported[14] that whole plasma, particularly heterospecific, produces increased Fe^{59} uptake and reticulocytosis in recipient animals because of a hemolytic reaction, rather than because of increased titers of EPF, when compared with boiled material The present long-term results (TABLES 9, 10 and 11) show that in the rat strain used here, whole plasma from one animal does not cause hemolysis when injected into another and that the increased erythropoietic potency of whole versus boiled plasma is real rather than simulated.

TABLE 9
COMPARISON OF 24-HOUR Fe^{59} UPTAKE AFTER 1 WEEK OF BOILED VERSUS UNBOILED PLASMA GIVEN I P DAILY

	Boiled	Unboiled
Phenylhydrazine anemic	12 9	38 1
Bled anemic	12 0	21 9
24-hour hypoxic	8 7	24 3
Control normal plasma	11 2	9 3

TABLE 10
COMPARISON OF HB AND HCT AFTER TWO WEEKS OF BOILED VERSUS UNBOILED PLASMA GIVEN I P. DAILY

	Boiled		Unboiled	
	Hb	Hct	Hb	Hct
Phenylhydrazine anemic	16 6	46 8	18 6	53 0
Bled anemic	16 4	47 0	16 6	49 0
24-hour hypoxic	15 9	42 6	16 8	46 6
Control plasma	15 9	43 8	15 6	41 0

TABLE 11
PERCENTAGE INCREASE* OF TOTAL RED CELL VOLUME IN HYPOXIC RATS INJECTED I P DAILY FOR TWO WEEKS

	Boiled	Unboiled
Phenylhydrazine anemic	22	43
Bled anemic	9	31
Hypoxic plasma	7	16

* As compared with controls receiving boiled and unboiled normal rat plasma Volume measurement done using Fe^{59}-labeled red cells

Chemistry of Erythropoietin

Although the exact nature of erythropoietin is still unsettled, the bulk of the evidence indicates that the humoral factor is polypeptidic in nature However, the possibility that the protein is serving as a carrier of low molecular weight molecules has not been eliminated. Our knowledge to date in this connection is summarized in TABLE 12.

Rambach *et al* [45] and Slaunwhite *et al.*[46] independently concluded that erythropoietin may be polypeptidic in nature, the former group also demon-

TABLE 12

Author	Assay	Method	Results and/or conclusions
Slaunwhite *et al.*	Fe^{59} uptake by erythrocytes	Physicochemical treatment of boiled anemic rabbit plasma	Activity stable to heat at pH 5.5 or 9, destroyed at pH 1 or 13, nondialyzable, digested by pepsin, trypsin and chymotrypsin, stable to mild oxidation and reduction, not extracted by chloroform
Rambach *et al.*	Hb, hematocrit, reticulocytes, Fe^{59} uptake by bone marrow erythrocytes, liver and spleen, P^{32} incorporation in DNA of spleen and bone marrow	Used heat stable EPF from plasma of anemic (O Mt NH_4) rabbits	α-Globulin fraction active as determined by paper strip electrophoresis of boiled anemic plasma per se, of EPF subjected to continuous flow electrophoresis and of NH_4SO_4 fractionation of whole anemic plasma
Gley and Delor	Increase in reticulocytes	Not given	Hematopoietin soluble in alcohol, $CHCl_3$, acetone, ether, thought to be triketoalcohol, certain steroids such as 11-dehydrocorticosterone and 21-desoxycortisone, give a hematopoietin response at low dose levels, hematostimulin a water soluble factor
Erslev and Lavietes	[illegible]	[illegible]	Globulin gave no re- [illegible]
Borsook *et al.*	[illegible] ticulocytes	[illegible]	[illegible] did not [illegible] liver extracts stimulating factor in plasma also requires amino acids and iron
Tohá *et al.*[51]	Recovery of Hb after bleeding	Not given, rabbit plasma used	Question significance of difference obtained
Linman and Bethell[11]	Increase in reticulocytes, erythrocytes & bone marrow activity	Injection of boiled anemic rabbit plasma extracts	EPF is nonprotein since heat or $HClO_4$ did not destroy activity
Van Dyke and Garcia	Not given	Not given, urine used	Urinary activity not ultrafilterable, not altered by washing with ether alcohol or by boiling, is destroyed by tryptic digestion, polypeptide

strating that the erythropoietic activity was associated with the α_2-globulin fraction. While most of the earlier work does not support this concept directly, it is in accord with it.

On the other hand, Gley and Delor[17] have postulated that there are two [illegible] hematostimulin is water-soluble and, to our [illegible] by Gley and Delor [illegible]

conclude that hematopoietin may be a triketoalcohol and, since it is a nonsaponifiable lipid, it may be a steroid. Consequently, they tested the effect of a number of steroids on reticulocytosis 11-Dehydrocorticosterone at a dose level of 15 μg in rat and 5 μg in the guinea pig and 21-desoxycortisone at 5 μg level in the guinea pig produced a two- to threefold increase in reticulocytes in 3 days A dose of 1 mg. of 11-desoxycorticosterone and cortisone was required to produce a 100 per cent increase. We have been unable to demonstrate reticulocytosis in rats administered for 14 days 1 to 10 mg of corticosterone intraperitoneally [48]

According to Bush,[49] the principal steroid produced by the rat adrenal is corticosterone, which is probably interconvertible with 11-dehydrocorticosterone The most amazing aspect of the reported phenomenon is the effectiveness of doses that are less than the natural secretion of the gland each minute Bush[49] reports 2.4 to 5.5 μg of α, β-unsaturated ketones/min for rats

One of the attractive aspects of a dual factor theory is that all the known properties of the erythropoietic factor or factors will fit one or the other of the postulated substances. Although this theory will not be discarded until an experimental basis exists for doing so, the remainder of this review will be in terms of erythropoietin.

Sometimes erroneously, Borsook *et al* [13] are credited with stating that erythropoietin is nonproteinaceous. Actually, they point out that their "nonprotein" plasma extract contains protein, and therefore they refrain from making any dogmatic statement concerning the nature of erythropoietin Similarly, in a subsequent paper they showed[50] that, although certain amino acids and iron will expedite the incorporation of C^{14}-leucine, C^{14}-glycine, C^{14}-histidine, or C^{14}-lysine into the proteins of rabbit reticulocytes, plasma supplies yet another unknown factor that still further increases incorporation

Linman and Bethell[11] concluded that erythropoietin was nonproteinaceous, since heat and perchloric acid treatment did not destroy its activity. Actually, their conclusion does not follow from their data, for many polypeptides or low molecular weight proteins such as ACTH, oxytocin, vasopressin, ribonuclease, and mucoproteins will withstand fairly vigorous treatment We regard the data of Linman and Bethell as in accord with protein nature

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tained both nonproteins and proteins, they could draw no conclusion as to the nature of erythropoietin Again, however, their data are in accord with the concept of Rambach

Van Dyke and Garcia[52] have experimented with urinary erythropoietin

IV-9, Freinkel *et al*[63] and Robbins and Rall,[64] and the binding of cortisol to transcortin (Sandberg and Slaunwhite,[55] Daughaday[56]). However, in the case of erythropoietin we do not believe this to be probable. This conviction is based on the enzymatic digestion reported by Slaunwhite *et al*[46] and Van Dyke *et al*[52] for, while enzymatic proteolysis would be expected to reduce or destroy the activity of a polypeptide, it should not alter the activity of a small molecule attached to it if the digestion mixture is injected directly into the test animal. It may be argued that the carrier protein is necessary for the maintenance of a constant level of erythropoietin, but since the rat, the usual test animal, has been demonstrated to possess erythropoietin, carrier will presumably be available.

If our hypothesis concerning the polypeptide nature of erythropoietin is correct, the course of future experimentation is clear. Unless a quantitative assay of erythropoietin requiring less material is discovered, however, progress will be slow and tedious.

Erythropoietic Activity of Plasma and Urine of Human Subjects

In view of the conflicting viewpoints concerning the chemical nature of erythropoietin and the varying methods of assay for this substance, it is not surprising that clinical studies have been rather unpredictable.[22, 65, 66] Levels of EPF in plasma extract from anemic patients have not correlated consistently either with the degree of anemia or with the etiology of the anemia (TABLE 13), that is, patients with similar degrees of anemia may show entirely different levels of EPF and, likewise, one patient with anemia due to acute leukemia may have a very high level of EPF in the plasma and another with anemia of the same etiology will have no elevation. Also, there has been no obvious correlation between the plasma EPF level and the presence or absence of a regenerative response of the erythron in a given patient. There may be a marked erythroid regenerative response in the absence of any elevation of plasma EPF and vice versa. Furthermore, serial studies in the same patient may show elevated levels at one time and normal levels at another, with no other obvious change in their clinical or hematological status. This is true of anemic patients, and also of those with polycythemia vera.

On the positive side it can be stated that the normal individual does not possess elevated levels of EPF in his plasma, whereas the anemic patient of varied etiology frequently does. Likewise, in polycythemia vera and secondary polycythemia elevated levels have been found in frequent instances and by differing methods of assay.[67, 68]

Recent studies from several laboratories [illegible]

[illegible]

then placed in 36/32 Nojax casings and dialyzed for 24 to 48 hours against running tap water. Some lots of urine were frozen initially and dialyzed at a later period. No significant differences in titers of erythropoietin were

TABLE 13

SHOWING DEGREE OF ANEMIA AND PLASMA TITER OF ERYTHROPOIETIC FACTOR IN EACH PATIENT

Pt	Blood	Diagnosis	Remarks	No. rats in group	Fe^{59} uptake				Reticulocytes		
					Mean	Range	S.E.	*P	Mean	Range	S.E.
V.N.	Hb 5.6 Hct 20.0	Ca of colon with retroperitoneal metastases		2	13.0	9.0–17.0	± 5.6	Insig	None		
J.B.	Hb 7.7 Hct 25.0	Chronic lymphatic leukemia	Depressed marrow erythropoiesis. Retics 5 per cent. Short RBC survival time	3	16.4	11.5–21.7	± 5.1	<1%			
A.C.	Hb 9.1 Hct 31.5	Ca of ascending colon		3	9.6	5.7–12.0	± 4.9	Insig			
A.B.	Hb 8.5 Hct 27.0	Ca of cervix-receiving radiation		3	26.1	15.3–36.8	±10.8	<1%			
M.Y.	Hb 9.0 Hct 24.0	Ca of cervix receiving radiation		3	19.6	10.5–26.9	± 8.3	<1%			
A	Normal human control			11	7.0	3.0–10.1	± 2.2	Insig.			
B	Saline control			2	7.5	5.3–9.7	± 3.1				
C.K.	Hb 10.0 Hct 31.0	Congenital spherocytic anemia	Increased marrow erythroid activity. Retics. 10 to 15 per cent. Short Cr^{51} RBC survival	3	7.9	2.8–12.8	± 5.1	Insig			
J.P.	Hb 6.2 Hct 18.0	Acute myeloblastic leukemia	Depressed marrow erythropos activity. Retics 1 per cent	4	44.3	37.8–52.1	± 6.0	<1%			
A	Normal human control			3	7.2	3.9–13.6	± 5.5	Insig			
B	Untreated control			3	5.9	2.5–11.9	± 5.2				
F.C.	Hb 8.5 Hct 27.0	Chronic lymphatic leukemia	Normal marrow erythroid activity. Retics 1.6 per cent. Sl. reduced Cr^{51} RBC survival	3	13.7	7.9–17.2	± 5.0	Insig	0.3	0.1–0.4	±0.22
G.R.	Hb 10.4 Hct 30.0	Chronic lymphatic leukemia	Evidence of increased hemolysis. Retics 5.5 per cent. Reduced Cr^{51} RBC survival	4	23.6	8.5–45.9	±17.0	Insig	1.4	0.4–2.3	±1.35
A	Normal human control			3	6.8	3.1–10.5	— 3.7	Insig	0.3	0.1–0.4	±0.22
B	Untreated control			3	3.4	2.6–3.8	— 0.7		0.1	0.1–0.1	±0.0

* The p values opposite each patient indicate a comparison between the patient and the normal human control group. Those opposite the control groups indicate a comparison between the controls themselves.

found in the fresh versus the frozen lots. Following dialysis, the urine samples were concentrated to one third their original volume, since this corresponded to the concentration factor we used with deproteinized plasma. However, there was a wide variation in the specific gravity of the 12-hour samples, indicating that the kidney had already introduced a wide variability in their concentration. For this reason it was thought to be more meaningful

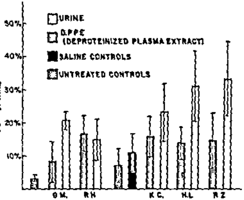


FIGURE 6. Comparative activity of normal human male urien versus D.P.P.E.

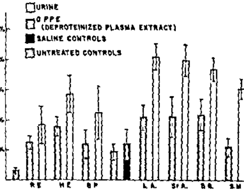


FIGURE 7. Comparative activity of anemic human urine versus D.P.P.E.

to concentrate all 12-hour samples to the same final volume. In this way variations due to dilution would be lessened. Therefore the urine was concentrated to 80 cc. in every instance, unless otherwise indicated. In some

The bio-assay technique was the same for both plasma extract and urine. Fourteen- to 20-day hypophysectomized rats were used. Two cc. of plasma extract or urine was given subcutaneously daily to each hypophysectomized rat for 3 days. On day 4, 1 μc. of Fe^{59} was given I.V. and on day 5 a 24-hour uptake was done.

The results are divided into several sections: (1) erythropoietic activity in urine of all subjects studied, TABLE 14; (2) comparative activity in deproteinized plasma extract and urine of normal and anemic subjects (FIGURES 6 and 7) and (3) the effect of the acidification and boiling procedure of Borsook on erythropoietic activity of urine (FIGURE 8). The vertical line at the top of the bars in FIGURES 6, 7, and 8 refers to one standard deviation. Acidification and boiling of the dialyzed urine by the procedure of Borsook had no

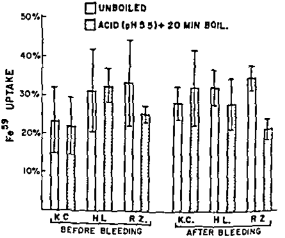


FIGURE 8. Effect of acidification and boiling on erythropoietic activity in urine of normal human males.

significant effect in 5 of 6 instances when a comparison was made. *In the* oth

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a. The advantages of the former in terms of simplicity are self evident. Serial studies of plasma and urine in several subjects will further clarify this relationship.

TABLE 14

ERYTHROPOIETIC ACTIVITY IN URINE OF NORMAL AND ANEMIC INDIVIDUALS

Pt	Blood		Diagnosis	No rats in group	Mean	Fe^{59} uptake range	S.E.	Per cent*
	Hb, gm	Hct %						
K C	18.5		Normal male	6	23.3	7.2–33.1	± 8.7	<1
H L	18.3		Normal male	5	31.1	14.4–40.6	± 10.6	<1
R Z	18.2		Normal male	6	33.3	17.2–43.7	± 11.1	<1
Untreated controls				5	7.1	2.2–13.6	± 5.2	<1
Saline controls				6	10.9	1.7–18.3	± 10.9	<1
A B	18.8		Normal male	5	23.8	15.6–28.9	± 4.9	<1
F D	17.5		Normal male	6	15.7	10.5–21.1	± 9.0	<1
F M	13.1		Normal male	6	21.9	17.7–28.7	± 4.3	<1
G M	16.3		Normal male	6	21.2	16.7–24.6	± 2.9	<1
T P	18.2		Normal male	6	21.1	15.8–25.2	± 4.4	<1
R H	17.9		Normal male	3	14.9	8.5–23.2	± 6.3	<1
Untreated controls				4	3.0	2.0–4.6	± 1.1	—
R D	15.7		Normal female	4	30.1	23.7–36.3	± 5.8	<1
H T	15.0		Normal female	4	24.8	10.0–31.4	− 9.9	<1
J S	15.1		Normal female	5	27.3	18.4–31.7	± 6.2	<1
Untreated controls				3	6.6	2.9–9.0	± 3.3	—
R F	8.5	23.0	Acute lymphoblastic leuk	6	18.4	10.2–27.6	± 6.2	<1
H E	6.5	21.0	Acute monocytic leuk	5	28.3	21.4–36.6	± 6.2	<1
B P	8.5	27.0	Carcinoma of Cx, grade IV, radiated	6	22.3	13.2–35.4	± 8.9	<1
M A	4.7	15.0	Acute lymphoblastic leuk	6	30.2	17.8–39.5	± 9.2	<1
J F	6.8		Carcinoma of bladder with metastases	6	33.4	23.8–42.0	± 6.7	<1
Untreated controls				4	3.0	2.0–4.6	± 1.1	-
L A	6.0	16.0	Carcinoma of breast with bone metastases	4	40.7	34.9–44.6	± 4.4	<1
Sr A	8.4	27.0	Carcinoma of bladder	6	39.8	30.3–44.6	± 5.2	<1
B G	6.6	21.0	Acute leukemia	5	36.8	31.7–42.8	± 4.0	<1
S M	6.9	22.0	Bleeding peptic ulcer	5	30.5	25.9–34.9	± 3.4	<1
Untreated controls				5	9.3	4.7–11.6	± 2.8	

The hemoglobin levels were determined by the method of Rimington.[41]

In our hands this method has given values in normals of 1.0 to 1.5 gm higher than the cyanmethemoglobin method.

Each of the groups separated by a double line was tested simultaneously.

It has been shown previously that the active erythropoietic-stimulating component in plasma extract is not dialyzable. The present results show that this is also true with human urine. These results are not in agreement with those of Hodgson and Tohá.[7] These investigators found that rabbit urine lost its erythropoietic activity after sixteen hours of dialysis. The reasons for this discrepancy are not apparent. Species differences and method of assay might be responsible.

Again, the variability of the EPF concentration in both normal and anemic subjects is evident. Although the urine of anemic subjects averages somewhat higher than that of normals, there is a considerable amount of overlap between the two groups.

Further progress in assessing the importance of EPF in clinical hematological problems appears contingent on several fundamental advances: (1) more accurate biochemical characterization of erythropoietin, (2) efficient and reproducible methods for its isolation from plasma and urine, (3) active extracts of known potency for testing purposes, and (4) uniform assay procedures so that results from one laboratory can be compared with those from another. In general, it would appear that assay in the intact animal over a term long enough to develop a true polycythemia is the most informative procedure. As the end point, one measures RBC, Hb, Hct, bone marrow erythroid activity, and TRCV. Although short-term assays such as Fe^{59} uptake and reticulocyte counts are simpler, occasionally they may be misleading. One may see increased reticulocytosis and Fe^{59} uptake within a few days following administration of certain extracts that are *not* invariably followed at a later date by significant polycythemia. Likewise, one may see a marrow erythroid response with no accompanying or subsequent true polycythemia. Such discrepancies dictate against using a single parameter as a reliable yardstick of erythropoietic activity. They also suggest that erythropoiesis is a complicated process involving many steps and that certain extracts may accomplish completion of some of these steps, but not all of them.

Summary

It has been shown (1) that the kidney plays a prominent role in the production of erythropoietin in response to anemic anoxia and cobalt administration, but not to hypoxic anoxia, (2) that hypophysectomized and binephrectomized rats produce erythropoietin in response to hypoxic anoxia, lending support to the thesis that there must be some source other than the kidney and pituitary for hypoxic anoxic erythropoietin, (3) that a too severe anoxia can inhibit erythropoiesis, (4) that neither the reticuloendothelial system nor the
b

erythropoietic activity of boiled versus unboiled plasma demonstrates that whole plasma is more potent than unboiled plasma, (9) that although the exact nature of EPF is still unsettled, the bulk of evidence indicates that the humoral factor is polypeptidic in nature, and (10) that normal human subjects do not possess elevated levels of EPF in their plasma, whereas some anemic patients of varied etiology do. It has been shown that urine frequently contains significant quantities of EPF, that a direct relationship between erythropoietic-stimulating properties of plasma extract and urine *appears* to exist, and that variability of EPF concentration in the urine of both normal and anemic subjects exists.

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EFFECT OF HEMOPOIETIN ON IRON METABOLISM IN NORMAL AND STARVED ANIMALS STUDIES WITH Fe^{59}

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This paper will discuss the effects of plasma and extracts of plasma and urine from anemic animals on iron metabolism and will comment on the various parameters that have been used to evaluate indirectly the intensity of erythropoiesis in assays of hemopoietin, in which Fe^{59} has been employed as a tracer.

Relation Between Plasma Iron Turnover, Plasma Iron, and Plasma Fe^{59} Disappearance Rate

When tracer doses of transferrin-bound Fe^{59} are injected into rabbits, the plasma radioactivity declines exponentially with time, and a plot of log. plasma Fe^{59} versus time gives a straight line[1] The slope of this line gives an estimate of the proportion of the plasma iron turnover per hour. In this report we shall refer to this parameter as K, the time constant of Fe^{59} disappearance from plasma FIGURE 1 shows the disappearance of tracer doses of Fe^{59} from plasma in a rabbit injected with normal rabbit's plasma and in a rabbit injected with plasma of phenylhydrazine-treated donors In interpreting the significance of the slope of these lines (K), it is necessary to know how K varies in animals with normal erythropoiesis, as adjudged by normal Hb concentration, reticulocyte count, plasma iron turnover, and incorporation of Fe^{59} into red cells FIGURE 2 shows the relation of the value of K and the plasma iron concentration in such animals The curve is a rectangular hyperbola of equation $K \times Fe = 1\ 24\ \mu g \times hour^{-1} \times ml^{-1}$ The dark points and open circles represent individual animals The dark points correspond to normal rabbits treated with 0 9 per cent saline solution or normal rabbits' plasma The circles represent results from normal rabbits injected with the supernatant from boiled plasma of phenylhydrazine-treated rabbits Previous experiments[2, 3] showed that the supernatant obtained

water extracts of the precipitated plasma proteins mixed with the supernatant The distribution of the points around the curve shown in FIGURE 2 is satisfactory enough to consider it a good representation of the relation between K and plasma iron This means that plasma iron turnover is independent of plasma iron concentration in the range of 1 to 3 2 $\mu g/ml$, and thus the value of K, the time constant of Fe^{59} disappearance, increases in proportion to the reciprocal of plasma iron concentration It is evident from this that the determination of disappearance rate of Fe^{59} alone is not a good measure of plasma iron turnover In FIGURE 2 the squares represent mean values of K for three different values of plasma iron in rabbits injected

with plasma and plasma extracts from phenylhydrazine-treated animal It is clear that injections of this material have shifted the points well abov the curve for normals and that, insofar as plasma iron concentration is con cerned, the points are displaced to the left. In this respect, it is suggesti that the point for the lowest iron concentration corresponds to three anima injected with extracts of boiled plasma, and that the mean for the highe iron concentration represents a group in which 8 of the 10 animals we injected with native plasma The square for 1.4 μg/ml. corresponds 6 animals, 3 of each group. The triangles represent mean values of K f

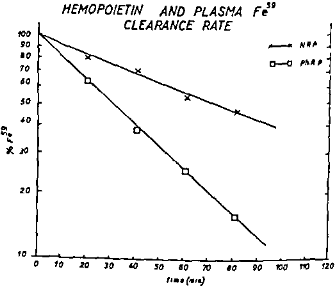


FIGURE 1 Disappearance of Fe^{59} from pla... from normal donors (N R P... donors (Ph R P) ... for two days Fe^{59} ...

3 different values of Fe^{59} in rabbits injected with plasma from severely bled donors or ... mean for ... normals ... poietic eff... plasma ir... with the "normal" population (dark points) Injection of dialyzed plasma from bled donors produced the results represented by the white triangle (9 animals) placed significantly above the curve and to the right of the native plasma group Finally, the white triangle with a dot in the center shows the result of an experiment with an acetone extract of plasma from bled donors (4 animals). The position is well below the reference curve and to the left of the native plasma group.

The percentage of injected radioiron appearing in the red cells (about 0 per cent) in the groups shown in FIGURE 2 does not differ significantly from ne group to another. There is one difference, however, in that the group njected with plasma from phenylhydrazine-treated donors showed maximum

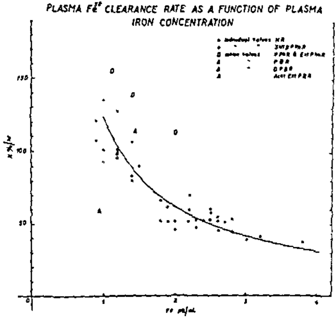


FIGURE 2. Relation between rate of plasma Fe^{59} clearance and plasma iron level in abbits. The following abbreviations are used: N R, normal rabbits; Snt B P Ph R, upernatant of boiled plasma from phenylhydrazine treated rabbits; P Ph R, plasma henylhydrazine treated rabbits; Ext P Ph R, water extract of boiled plasma from henylhydrazine treated rabbits; P B R, plasma-bled rabbits; D P B R, dialyzed plasma led rabbits, and Acet. ext. P B R, acetone extract plasma bled rabbits.
The dose of solutions injected was 10 ml/kg twice a day for 2 days. Fe^{59} was injected 6 hours after last injection.

ptake at 24 hours whereas, in the other groups, the maximum of Fe^{59} in red ells was reached at 48 to 72 hours after tracer injection.

Plasma Iron Turnover, Plasma Iron Concentration, Plasma Fe^{59} Disappearance Rate, and Fe^{59} in Erythrocytes of Starved Animals

FIGURE 3 shows the value of K, plasma iron concentration, plasma iron

boiled plasma of phenylhydrazine-treated donors produce a definite increase

in plasma iron turnover and a drop in plasma iron, as in normal animals The increase of percentage Fe^{59} appearing in erythrocytes is striking, an effect that is not apparent in normal animals, since in these the fraction

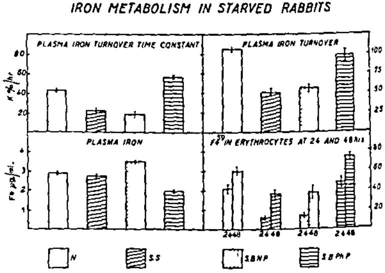


FIGURE 3 Mean values for plasma Fe^{59} clearance rate, plasma iron, plasma iron tur[illegible] over, and Fe^{59} in red cells of rabbits fasted for 6 days The solutions were injected on t[illegible] third and fourth days of fasting (10 ml intravenously twice a day), Fe^{59} was given 16 hou[illegible] after the last injection

The following abbreviations were used for the four groups N, normal rabbits, S[illegible] starved rabbits injected with saline, S B N P, starved rabbits injected with extract [illegible] boiled plasma from normal rabbits, and S B Ph P, starved rabbits injected with extra[illegible] of boiled plasma from phenylhydrazine-treated rabbits

appearing in the noninjected controls is already high (80 per cent) Extrac of boiled plasma from normal rabbits show no effect.

Plasma Iron and Fe^{59} in Liver and Erythrocytes of Starved Rats

FIGURE 4 summarizes the effects of extracts of plasma from phenylhyd zine-treated donors on plasma iron concentration of starved rats. Plasr

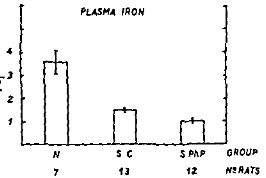


FIGURE 4 Plasma iron levels in normal and fasted rats The animals were fasted for

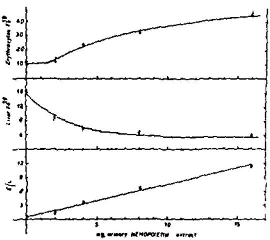


FIGURE 5 Fe^{59} in erythrocytes and liver and erythrocyte liver ratio 24 hours after tracer injection in starved male rats injected with different doses of urinary hemopoietin preparations The rats were fasted for four days Hemopoietin was injected on the second and third day of fasting and on the morning of the fourth day, prior to Fe^{59} injection

determination of relative specific activity of extracts FIGURE 6 shows the effects of some active urinary fractions,[4] compared with the effects of the same dose of the crude alcoholic extract of urine used as starting material. The higher specific activity of the fraction obtained on raising the alcohol concentration from 50 to 80 per cent at *p*H 4 5 is shown clearly.

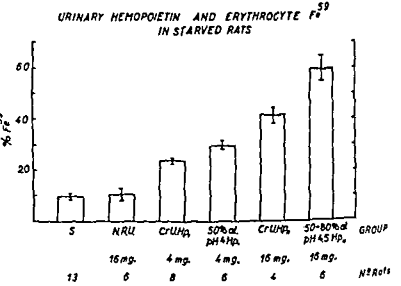


FIGURE 6 ... doses of ... urinary hem... ...ted in ... ract of
FIGURE 5 ... ...r cent ... normal rabb... ...ohol at ... nt M. ... alcohol

Discussion

The experimental results presented show that plasma iron turnover in animals with normal erythropoiesis is independent of plasma iron concentration over wide ranges. This suggests that the bone marrow works with iron concentration in the region of excess substrate in relation to the enzyme(s) involved in the incorporation of Fe into Hb. This relationship between plasma iron concentration and turnover in rabbits confirms the findings of Bothwell *et al.*[9] and demonstrates that the rate of disappearance of Fe^{59} from plasma, used alone, is not a valid criterion for Hb synthesis.

The effects of extracts of plasma obtained from anemic rabbits on iron metabolism of normal and starved animals show that plasma of anemic animals not only contains factors that influence erythropoiesis, but also affects iron metabolism per se, not just as a consequence of the modification of Hb synthesis. Plasma from bled animals appears to contain a mixture of substances, some of which lower plasma iron and possibly depress erythropoiesis, and another that stimulates Hb synthesis. Plasma from phenylhydrazine-treated animals, which contains high amounts of hemopoietin, appears to have a factor(s) that tends to maintain iron level in the face of increased bone marrow demands, for the extracts obtained by boiling the plasma, although conserving their effect on plasma iron turnover, produces a

clear drop in plasma iron concentration The experiments with the supernatant and extracts of boiled plasma suggest that the observed drop in plasma iron is caused by a factor other than hemopoietin, for injection of the supernatant produces a drop in plasma similar to that seen with the extract, and yet does not affect plasma iron turnover

Knowledge of factors controlling iron levels is too scarce at present to allow much discussion, but the results presented in this paper are indicative of the existence of a controlling system of a positive and negative nature

The results of tests in starved receptors confirm previous findings[6, 2] as to the sensitivity of animals with depressed erythropoiesis to hemopoietin, and show that quantitative bio-assays are feasible and that chemical fractionation procedures for hemopoietin can be carried out employing the criterion of specific activity as an index of purification[4]

Summary

Plasma iron turnover is shown to be independent of plasma iron concentration in rabbits Plasma from phenylhydrazine-treated and bled donors contains a factor (hemopoietin) stimulating Hb synthesis It is probable also that the plasma of anemic animals contains a factor(s) other than hemopoietin that influences plasma iron level

Hemopoietin injected into starved animals produces a notable increase in iron turnover and marked changes in the distribution of iron between marrow and depots An example is given of the use of starved rats for quantitative bio-assay of hemopoietin obtained from the urine of phenylhydrazine-treated donors.

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OBSERVATIONS ON THE PHYSIOLOGY OF ERYTHROPOIETIN AND ITS ROLE IN THE REGULATION OF RED CELL PRODUCTION

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It has been demonstrated by a number of laboratories[1] that, following exposure to acute hypoxia, whether due to anemia or decreased oxygen pressure (pO_2) of the inspired air, erythropoietin is released into the plasma. The rate at which erythropoietin is released and the maintenance of plasma levels under conditions of chronic hypoxia have not been studied extensively. Prentice and Mirand[2] observed a substantial increase in erythropoietin after 24 hours of exposure to 10 per cent O_2, but not after 48 hours except in the presence of liver damage. In contrast, Erslev[3] noted similar activity after 20 and 48 hours of anemia or exposure to 10 per cent O_2.

It becomes of importance, particularly in considering the role of erythropoietin in clinical disorders, to determine the rate of formation and removal of erythropoietin from the plasma, the influence of the bone marrow and renal excretion on the plasma erythropoietin level, and the role of hypoxia and erythropoietin in the response to posthemorrhagic anemia.

Method of Study

Erythropoietin production was stimulated by exposure of Sprague-Dawley rats at a barometric pressure of 305 mm of Hg (23,000 feet) or by bleeding as previously described.[4] In bled animals in which the effect of hypoxia was studied, one group of animals was bled and was kept at normal atmospheric conditions while the other group was placed in a chamber containing a 50 per cent O_2 and 50 per cent N mixture that was exchanged under positive pressure at a rate of 2 l./min.

Plasma from patients was prepared for testing by diluting with 3 volumes of H_2O, adjusting the *p*H to 5.5, and boiling for 5 min.[5] The supernatant was removed by centrifugation, dialyzed against 4 to 5 changes of distilled H_2O, lyophilized, and resuspended in a *p*H 7, phosphate-buffered isotonic saline. Since this method of plasma preparation results in the destruction of 70 to 90 per cent of the erythropoietin,[6] the plasma was concentrated sevenfold prior to testing. Human urine was prepared by dialyzing against 5 to 6 changes of distilled H_2O, lyophilizing, and resuspending in phosphate-buffered saline. A volume equivalent to 20 per cent of the 24-hour output

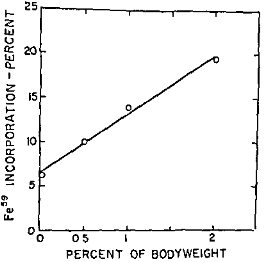


FIGURE 1 The relationship of the amount of injected erythropoietin to the erythropoietic response in fasted animals. Erythropoiesis was measured by Fe^{59} incorporation. Erythropoietin was obtained by exposing rats at 305 mm of Hg (23,000 feet) for 16 hours. Each point represents the mean of 6 animals.

correlation with the Fe^{59} incorporation, therefore, subsequently the Fe^{59} incorporation alone was used. Moreover, there also appeared to be a rough dose-response relationship in terms of Fe^{59} incorporation (FIGURE 1).

Results

Rate of appearance and disappearance of erythropoietin. Erythropoietin was demonstrable within 2 hours of exposure to reduced barometric pressure. Thereafter there was a progressive increase in the erythropoietic activity of plasma until a peak was achieved following 16 to 24 hours of continuous exposure (FIGURE 2). Following more prolonged exposure, the plasma level of erythropoietin was not maintained. In each of 8 experiments in which the plasma erythropoietin level observed at 16 to 24 hours was compared with that observed at 48 hours, the 24-hour level was substantially greater than that found at 48 hours (FIGURE 2). When animals were exposed for as long as 90 hours, erythropoietin could no longer be demonstrated consistently.

In rats in which the release of erythropoietin was evoked by the removal of approximately 30 per cent of the blood volume in a single bleeding, the erythropoietin levels were substantially less than those observed in altitude-exposed animals. The Fe^{59} incorporation of animals given anemic plasma varied from 11 to 14 per cent, in contrast to the 22 to 30 per cent values observed after the administration of altitude plasma. As was the case with altitude, plasma collected 48 hours after the induction of anemia had less erythropoietin than did that collected at 24 hours. In 4 experiments these

values ranged from 11 to 14 per cent for plasma collected 24 hours after the induction of anemia and from 8 to 11 per cent in the 48-hour plasma (normal plasma 5 to 8 per cent)

Disappearance times of erythropoietin were determined by exposing animals at simulated altitude for a period of 16 hours, after which they were returned to normal atmospheric con... les collected at varying intervals for the determin... may be seen in FIGURE 3 that under... of erythropoietin is curvilinear, and that some activity... strable 12 hours after discontinuance of the exposure. It is recognized

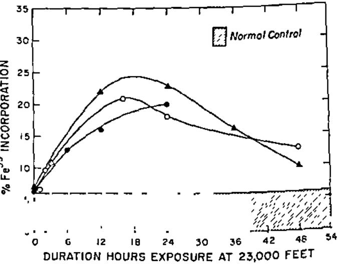


FIGURE 2 The relationship of plasma erythropoietin levels to duration of exposure a[t] a simulated altitude of 23,000 feet Each point represents the mean of 6 animals Th[e] different symbols indicate different experiments

that initially there may have been some overlap between production an[d] utilization or inactivation, but it seems unlikely that this extended beyon[d] the first hour or two

Relationship of Degree of Hypoxia to Plasma Levels of Erythropoietin

It has been demonstrated previously that the magnitude of the erythr[o]poietic response to acute exposure to hypoxia is dependent upon the degr[ee] of hypoxia [8] Thus, the plasma from rats exposed at 450 mm. of Hg produce[s] ... fro[m]

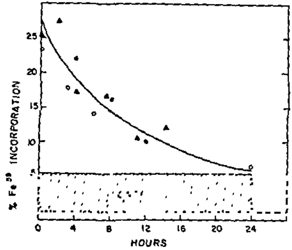


FIGURE 3 The rate of disappearance of erythropoietin from the plasma Donor animals were exposed at a simulated altitude of 23,000 feet for 16 hours after which they were returned to atmospheric conditions and plasma was collected at the intervals indicated on the abscissa Each point represents the mean of 6 recipient animals Each symbol indicates a different experiment

TABLE 1
RELATIONSHIP OF SEVERITY OF ANEMIA TO ERYTHROPOIETIN LEVEL IN CHRONIC ANEMIA

Fe[59] incorporation*		Hct†	p‡
Normal plasma	Anemic plasma		
7 ± 1 03	13 ± 0 53	25	0 01
5 ± 0 55	12 ± 0 81	26	0 01
6 ± 1 19	10 ± 1 08	29	0 03
8 ± 1 16	9 ± 0 97	32	n s
8 ± 1 00	9 ± 1 00	34	n s

* Fe[59] values given are mean values (6 rats) ± S E in fasted rats given 2 injections of boiled anemic dog plasma

† Refers to hematocrit of donor

‡ The probability that observed difference occurred by chance, using one-sided t test

concentration of a boiled alcoholic extract[8] was prepared from dog plasma and tested in rats It is evident that in the presence of substantial anemia, hematocrit 20 to 25, there is an unequivocal response In contrast, erythropoietin could not be demonstrated in the plasma of animals with chronic anemia in which the hematocrits were in the range of 30 to 35.

Utilization of Erythropoietin by Bone Marrow

Since plasma erythropoietin levels decreased in the face of continued hypoxia, where the only substantial change appeared to be that of an increasing cellularity of the marrow, the relationship of bone marrow activity to plasma erythropoietin was determined. Bone marrow hypoplasia was induced by a sublethal dose of ionizing radiations (400 r)* at intervals of 0 to 48 hours prior to exposure of the animal to reduced barometric pressure (300 mm Hg) Plasma erythropoietin levels were determined after periods

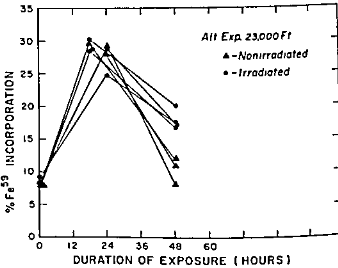


FIGURE 4 Comparison of plasma erythropoietin levels in irradiated and normal animals exposed simultaneously to a simulated altitude of 23,000 feet Each point represents the mean of 6 animals

varying from 12 to 90 hours of continuous exposure and compared with simultaneously exposed, nonirradiated animals. The plasma levels of erythropoietin after 24 hours of exposure were similar in both groups However, at 48 hours the irradiated animals had a substantially greater amount of plasma erythropoietic activity than did the nonirradiated altitude-exposed animals (FIGURE 4) A similar difference was noted in animals exposed continuously for 90 hours (FIGURE 5) Disappearance curves determined on 3 occasions after discontinuance of a 16-hour exposure showed similar differences (FIGURE 6) A crude approximation of the half-disappearance time in the nonirradiated group was 3 to 5 hours, in the irradiated group it ranged from 5 to 10 hours

* Radiation was delivered using a 2 5 mev Van de Graaff generator at an average dose rate of 50 r/min measured with a Bendix ionization chamber known to be air-equivalent at this energy

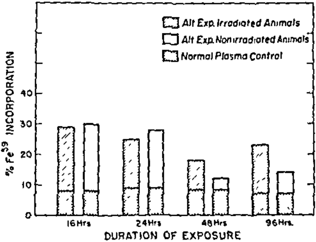


FIGURE 5 Comparison of plasma levels of erythropoietin in irradiated and normal animals exposed simultaneously to a simulated altitude of 23,000 feet Each bar represents the mean of 6 animals The differences between irradiated and nonirradiated animals at 48 and 90 hours are statistically significant

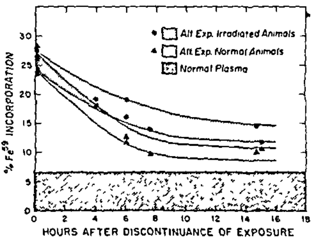
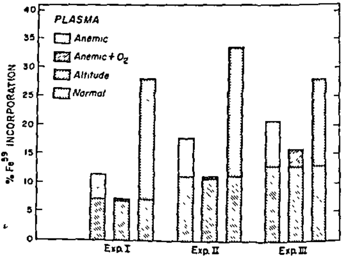


FIGURE 6 Comparison of rate of disappearance of erythropoietin from the plasma of normal rats and those with radiation induced hypoplasia Animals were exposed for 16 hours at 23,000 feet and then returned to atmospheric conditions when plasma was collected at the intervals indicated on the abscissa Each point represents the mean of 6 animals

Role of Hypoxia in the Erythropoietic Response

Generally, it has been conceded that anemia produces an increase in red cell production as a consequence of the decrease in O_2-carrying capacity of the blood, with resultant tissue hypoxia and liberation of erythropoietin. To determine if this was the sole mechanism involved, reticulocyte and erythropoietin levels of rats bled and exposed to 50 per cent O_2 for 24 hours were compared with those of bled anemic animals that remained at atmospheric conditions. In 3 experiments in which this comparison was made,



represents the mean of 6 determinations in fasted animals

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with those of control animals receiving normal plasma (FIGURE 7).

Despite the decrease in erythropoietin resulting from the administration of 50 per cent O_2, the reticulocyte response of the donor animals was not

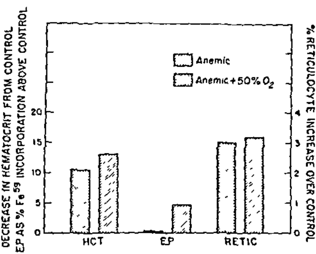


FIGURE 8 The fall in hematocrit reticulocyte count and erythropoietic level of bled rats compared with those bled and placed in an atmosphere of 50 per cent O_2. The values were obtained 24 hours after the production of anemia. Plasma was pooled prior to testing for erythropoietin.

Clinical Observations

In considering the physiological role of erythropoietin, certain clinical observations appear pertinent. Increased erythropoietin levels have been observed in a variety of clinical states. As might be anticipated from the experimental studies, the level of erythropoietin appears to be a function of the severity of anemia and the degree of bone marrow activity.

The contrast of the erythropoietin levels in sickle cell anemia and certain instances of refractory anemia offers a good illustration (TABLE 2). The plasma from one patient with sickle cell anemia, in whom the hemoglobin was 6.6 gm., the reticulocyte count 18 per cent, and with 20 nucleated RBC/100 WBC, increased the Fe^{59} incorporation of the assay animals from 7 to 10.5 per cent. In a second case of sickle cell anemia in which the hemoglobin was 8 gm. per cent, the plasma on two occasions produced an increase in Fe^{59} incorporation to 10 per cent from that of 8 per cent observed with normal plasma. In contrast, in one patient with a chronic refractory anemia, a hemoglobin of 9.5 gm. per cent, and a morphologically normal marrow, unconcentrated plasma increased the Fe^{59} incorporation from 7 to 14 per cent. Even more striking were the results achieved with plasma from a patient with an aplastic marrow and a hemoglobin of 5 gm. per cent. The concentrated plasma from this patient increased the Fe^{59} incorporation from the control (normal plasma) of 7 per cent to a level of 50 per cent. Intermediate between these cases is one of a patient with an acquired hemolytic anemia associated with chronic lymphocytic leukemia. The plasma of the patient

was tested for erythropoietin at a time when the hemoglobin was 6 gm per cent, with a reticulocyte count of 6 per cent It is to be noted that this patient had infiltration of the marrow with leukemic cells and that his erythroid response as judged clinically by transfusion requirements and by reticulocyte count was suboptimal, indicating a production rate of only two- to threefold of that observed in a normal individual. This patient's plasma, when concentrated, increased the Fe^{59} incorporation of fasted animals to 21 per cent

TABLE 2
RELATIONSHIP OF ERYTHROPOIETIN TO HEMOGLOBIN AND BONE MARROW FUNCTION IN SELECTED CLINICAL CASES

	E.P.*	Hb (gm. percentage)	Retic (percentage)
Control	5-8	—	—
Normal plasma	5-9	14-15	0.5-1.5
Sickle cell anemia	10	8	31
Sickle cell anemia	10	7	14†
Acquired hemolytic anemia with chronic lymphatic leukemia	21	6	6-7
Refractory anemia	14	9.5	1-2
Aplastic anemia	49	4.2	0.5

* Erythropoietin measured as mean Fe^{59} incorporation in fasted rats
† Fourteen nucleated red cells/100 W.B.C.

It has been possible to demonstrate significant urinary levels of erythropoietin only in those instances where there is substantial plasma activity. Thus, urine from the patient with refractory anemia increased Fe^{59} incorporation from 9 to 18 per cent That of the patient with lymphatic leukemia and hemolysis increased Fe^{59} incorporation to 15 per cent, while that from patients with sickle cell anemia was without demonstrable effect. The relationship of the urinary factor to hypoxia was shown by transfusing a patient to normal levels and testing urine before and after transfusion The pretransfusion level of 18 per cent was reduced in 24 hours to the control values of 9 per cent

Discussion

The data presented here demonstrate that, following an acute exposure to hypoxia, there is a prompt release of erythropoietin into the plasma The plasma level continues to increase until a maximum is achieved between 16 and 24 hours; thereafter the plasma level declines The level at which

It is evident from the high levels of erythropoietin in animals with radiation-induced hypoplasia that bone marrow utilization accounts, at least in part, for the clearance of plasma erythropoietin. Thus, for any given level of hypoxia one would anticipate that the state of bone marrow function would determine the degree of elevation of erythropoietin. Of course, this would obtain only if the mechanism for production of erythropoietin remained intact. For example, in chronic renal disease with azotemia there is a suppression of erythropoietin production.[9]

These considerations would explain both the clinical and experimental observations presented, as well as those reported by others.[1] In chronic hemolytic anemia due to an abnormal hemoglobin, in which the hemoglobin was in the range of 7 to 10 gm. and was associated with intense erythroid hyperplasia, only a slight elevation of erythropoietin was observed. In contrast, in a patient with hemolytic anemia associated with chronic lymphatic leukemia, in which the hemoglobin of 6 gm. was associated with only mild erythroid hyperplasia as evidenced by a 6 per cent reticulocyte count, a more substantial elevation in erythropoietin was noted. Similarly, in dogs with chronic blood loss anemia in which the iron levels were maintained, increased erythropoietin was observed if the hematocrit was in the range of 20 to 25, whereas at levels of 30 to 35 no increase in erythropoietin could be demonstrated. In contrast, in a patient with refractory anemia and a hemoglobin in the range of 9 to 10 gm. a substantial elevation of erythropoietin was readily detected.

It may be recalled that, in attempting to demonstrate erythropoietin in the plasma of animals with phenylhydrazine-induced anemia or chronic blood loss anemia in cross species experiments, it has been necessary to use animals with hematocrits in the range of 10 to 20.[3, 16] Ersley[3] has reported substantial erythropoietin in the plasma of rabbits 48 hours after the induction of anemia. However, in all instances the hemoglobin was less than 7 gm per cent. Similarly, in clinical reports by Piliero et al.,[11] Guerney et al.,[12] and Prentice and Mirand[13] there has been a good correlation between the level of erythropoietin and the severity of the anemia and the state of erythroid function of the donor marrow.

The fate of erythropoietin once liberated is in part, as already discussed, a function of marrow utilization. In addition there must be other pathways of erythropoietin removal, since erythropoietin disappearance has already been demonstrated in the virtual absence of functioning erythroid tissue, as is seen in irradiated animals or following hypertransfusion in patients with refractory anemia. Clearly, renal excretion is a potential removal mechanism. Its demonstrated association with high plasma levels suggests the presence of a threshold for excretion. Another pathway has been suggested by Jacobsen and Alpen[19] and Prentice and Mirand.[7] These authors found increased plasma erythropoietin levels in hypoxic animals with coexistent liver damage. At present the evidence for this appears inconclusive.[8] It is clear from the foregoing that the problem of erythropoietin metabolism requires further exploration before definitive conclusions may be drawn.

Generally it is considered that the state of tissue oxygenation controls the

release of erythropoietin This is based upon the observation that erythropoietin is released in response to a decrease in O_2 capacity or pO_2 The observation that the erythropoietin content of bled animals is reduced when they are placed in an atmosphere of 50 per cent O_2 is in accord with this view

The failure to abolish the reticulocyte response in bled animals placed in a high O_2 atmosphere raises the question as to whether hypoxia is the sole determinant of red cell production Reinhard *et al.*[14] and Tinsley *et al.*[15] have demonstrated clinically that the administration of high concentrations of O_2, 70 to 90 per cent, is associated with a depression, but not abolition, of the erythropoietic response in patients with sickle cell anemia, in hereditary spherocytosis, and in normal individuals, and that it results in a suboptimal, but definite response to liver therapy. In their studies, an increase in alveolar pO_2 of three- to fivefold was observed Since the arterial pO_2 approximates that of the alveolar under these conditions[16] and is reflected by a substantial rise in tissue pO_2,[17] one may assume that hyperoxia existed in the tissues in these studies. However, as in our studies, the block of red cell production was by no means complete In normal individuals, Tinsley *et al.*[15] observed [illegible] d minished

locyte production by hypertransfusion of normal mice.

There are several other clinical and experimental observations that argue against the unified concept advanced by Fried *et al.*[7] that the relationship of oxygen demand to supply governs the rate of red cell production It has been observed that in normal individuals daily fluctuations in plasma volume may produce alterations of as much as 8 per cent in the hemoglobin concentration of blood, although the red cell and hemoglobin mass remain constant[18] In view of this, it is difficult to ascribe to tissue hypoxia the increase in red cell production necessary to compensate for minor blood loss such as may be incurred from gingival or hemorrhoidal bleeding, minor lacerations, or the removal of 5 to 10 cc for blood samples

The compensated hemolytic syndromes, such as are commonly seen in hereditary spherocytosis, offer another argument against hypoxia as the regulant It may be suggested that, while the hemoglobin concentration is within the normal range in such patients, the value is lower than the individual's true genetic normal requirement Indeed, splenectomy in some [illegible] ntra- [illegible] shed [illegible] tient

had a hemoglobin that varied from 13 to 13 8 gm. per cent, reticulocytes, 150 to 225,000/cu mm , indirect bilirubin, 4 mg per cent, fecal urobilinogen 380 mg /day; and Cr^{51} T ½, 14 days This patient had a partial exchange transfusion in which 1500 cc of her blood was removed and replaced with 1500 cc. of normal blood. In the 100 days following the transfusion, the hemoglobin remained in the range of 13 to 13 8 and the mean values were slightly lower than those observed prior to transfusion. The reticulocytes

decreased immediately after transfusion and in the first 30 days ranged from 85 to 120,000/cu mm Thereafter, there was a gradual rise to the pretransfusion level One cannot explain the compensated state of this patient on the basis of hypoxia Had this been the case, the decrease in the rate of destruction due to replacement by normal cells should have resulted in an increase in hemoglobin without change in the number of reticulocytes

Observation of the response to hemolysis following insertion of a plastic valvular prosthesis in the circulation[20, 21] has afforded us an opportunity to observe compensated hemolysis in which the normal hemoglobin level was established prior to the onset of hemolysis

TABLE 3
RED CELL VALUES IN DOGS WITH COMPENSATED HEMOLYSIS FOLLOWING INSERTION OF A VALVULAR PROSTHESIS

Dog	Preoperative values		Postoperative range*		RBC survival† (days)
	Hct (percentages)	Retic (percentages)	Hct	Retic (percentages)	
S93	45	0.3–0.3	45–47	0.4–1.3	20
S102	49	0.1	46–50	0.4–2.1	17
S13	53	0.4–0.7	49–55	0.7–2.8	
S88	42	0.2	42–45	0.5–0.9	16
S47	48	0.5	50–56	0.8–1.4	17

* Only values obtained after the twentieth postoperative day are included
† Fifty per cent disappearance time Dogs 93, 88, 47 measured with Cr^{51}, normal range 21 to 27 days, 102 measured with diisopropyl fluorophosphate (DFP^{32}), normal 45 to 50 days

One patient in whom a plastic ball valve prosthesis of the Hufnagel type[22] was inserted into the descending aorta for the correction of aortic insufficiency had a preoperative hematocrit of 43 Two years later his hematocrit varied between 42 and 44 during a time when the red cell survival, determined by the technique of differential agglutination, indicated the presence of a random loss amounting to 1.5 per cent/day in addition to the normal loss from senescence In TABLE 3 data are presented on 5 normal dogs in which a plastic bypass of the aortic valve had been inserted This prosthesis is associated with variable hemolysis, the determinants of which are the type of valve and the rate of change of the pressure gradient across the valve, which governs the impact force on seating[21] In some a compensated hemolytic state results TABLE 3 presents pertinent data collected 20 days to 1 year after operation in 5 such dogs It is to be noted that the postoperative hematocrit range is identical with or slightly higher than the preoperative value Increased reticulocytes and a shortened cell survival furnish evidence for the hemolytic process It may be seen, then, that these animals compensate for an increased rate of destruction in the absence of hypoxia

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There are several other clinical and experimental observations that argue against the unified concept advanced by Fried *et al.*,[7] that the relationship of oxygen demand to supply governs the rate of red cell production. It has been observed that in normal individuals daily fluctuations in plasma volume may produce alterations of as much as 8 per cent in the hemoglobin concentration of blood, although the red cell and hemoglobin mass remain constant.[19] In view of this, it is difficult to ascribe to tissue hypoxia the increase in red cell production necessary to compensate for minor blood loss such as may be incurred from gingival or hemorrhoidal bleeding, minor lacerations, or the removal of 5 to 10 cc. for blood samples.

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S13	53	0 4-0 7	49-55	0 7 2 5	—
S88	42	0 2	42-45	0 5-0 9	16
S47	48	0 5	50-56	0 8-1 4	17

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The differences between the response to anemia and decreased arterial pO_2 are likewise of interest. It has been shown (FIGURE 7) that the increase in erythropoietin in response to the reduction of arterial O_2 saturation incident to exposure at 23,000 feet is substantially greater than that associated with the withdrawal of 30 per cent of the blood volume. Presumably this is the consequence of a partial compensation by the anemic subject through an increase in cardiac output[22] and an increase in the coefficient of O_2 utilization, that is, the extraction rate of O_2.[24] Nevertheless, the increase in red cell production in the anemic subject, as measured by reticulocytosis and increase in hemoglobin mass, is equal to or greater than in those exposed to decreased arterial pO_2. In phenylhydrazine-induced anemia the entire red cell population may be replaced in 2 weeks,[9] in hemolytic disorders increases of cell production of sevenfold have been reported[24] whereas, in adaptation at altitude a somewhat slower, more gradual rise in hematocrit associated with a two- to threefold increase in Fe^{59} utilization[25] is seen. In part this may reflect the state of oxygenation of the marrow itself, red cell production being limited by severe hypoxia.[8] It might be suggested, however, that more than one mechanism is responsible for the observed increase in erythropoiesis in the anemic state. This contention is supported by the observations of Altland and Parker on the box turtle.[26] They noted a substantial reticulocytosis following blood loss, but not when the turtles were intermittently exposed to reduced barometric pressures equivalent to a simulated altitude of 35,000 to 45,000 feet, even though the metabolic rate was sustained. At these levels of altitude the pO_2 of the inspired air is 37 and 23 mm of Hg, respectively. McCutcheon[27] has demonstrated that a pO_2 of this magnitude is associated with a hemoglobin saturation of 60 to 75 per cent, so that it can be assumed that the turtles in altitude experiments were, in fact, hypoxic.

The above considerations lead us to conclude that the regulation of erythropoiesis cannot be explained solely on the basis of erythropoietin release in response to a tissue oxygen supply-demand relationship. In the search for an alternative explanation we have proposed elsewhere[1] a modification of the inhibitor theory that has been advanced in other areas of cellular control ...
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and acts directly on the bone marrow. Bleeding, no matter how slight, by removing a proportion of older cells would reduce the number of cells destroyed per day and the amount of inhibitor released, hemolysis would result in a ...
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poietin mechanism would afford a booster in the event of more substantial demand. Thus far our attempts at experimental verification have proved unsuccessful.[9] It is recognized also that the withdrawal of even small amounts of blood by removing cells of all ages would result not only in an immediate reduction of old cells but, in view of the similar reduction in younger cells, a deficit of cells entering old age would persist throughout the

entire life span of red cells. Thus, the mechanisms involved in the regulation of red cell production remain in question. The evidence presented, however, strongly argues against a unified mechanism based on hypoxia and in favor of a dual control of erythropoiesis.

Summary

Data have been presented to show that plasma erythropoietin levels are influenced by the functional state of the erythroid tissue of the marrow, as well as the severity of the hypoxic stimulus. In clinical disorders this relationship has provided an explanation for the high plasma levels in refractory anemias and the relatively low values in most hemolytic disorders.

Consideration has been given to the role of erythropoietin in the regulation of erythropoiesis. Arguments have been presented to support the contention that accelerated erythropoiesis can be produced in the absence of hypoxia, indicating that a second mechanism operates in the control of erythropoiesis.

Acknowledgments

I thank Howard Andrews of the National Cancer Institute for the use of his irradiation facilities, and for the measurements of field uniformity and delivered dose which were critical for the reproducibility of these experiments. I am indebted also to Paul Altland and Heinz Specht of the National Institute of Arthritis and Metabolic Diseases, who generously provided access to the decompression chamber used in these studies.

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A DISCUSSION OF HUMORAL ERYTHROPOIETIC FACTORS*

Henry Borsook

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I propose to discuss the evidence for the existence of specific humoral erythropoietic factors, with some attention to the question of whether there may be more than one such factor, the matter of producing a potent extract from the blood, and some physical and chemical properties of preparations with erythropoietic activity. To facilitate this discussion, I shall designate the erythropoietic material in plasma as EP and that in urine as EU. EP and EU may or may not be the same, there may be more than one EP or EU.

It will help to eliminate some of the disagreement in the literature if we accept the following working hypothesis. The responses to increasing potency of EP or EU appear in the following order: increased red cell activity in the bone marrow, reticulocytosis, increased uptake of Fe^{59} in the blood by an increase of newly formed red cells, increased red cell volume, hematocrit, red cell count, and hemoglobin. Only powerful stimulation can provoke a *true polycythemia in the 10 days to 2 weeks of the usual experiment, then* all the signs of increased erythropoiesis are present. It does not follow, and it is not the case, that a preparation causing a reticulocytosis or even an increased uptake of Fe^{59} will also induce polycythemia. Herein lies much of the contradiction in the literature. It may be that there are several physiological erythropoietic factors and that the attack of some of these is more deep-seated than others, that is, some may cause only a reticulocytosis, and the resulting increased red cell formation is too slow and too small to be noticed except by tracer methods such as Fe^{59}. Also, it may be that there is but one erythropoietic factor, it can stimulate a true polycythemia if a sufficiently large dose is used, but a preparation of low potency may cause only a reticulocytosis or small increase in uptake of Fe^{59}. Elsewhere in this monograph, Hodgson presents data showing that the relation of the response to dose is *logarithmic*. This would be sufficient to account for the difficulty of producing a polycythemia with the plasma after hemorrhage or phlebotomy, because the usual anemia after hemorrhage elicits only a moderate increase in EP (see below).

The modern era begins with the often quoted paper by Carnot and Deflandre.[7] In a typical experiment these investigators withdrew 30 ml of blood from a rabbit, bled the animal again on the next day, and injected 9 ml of the serum of that blood into a normal rabbit, whose red cell count rose 24 hours later from 5.5 million to 8 million/cu mm. They called the substance in the anemic serum that induces the polycythemia hemopoietin

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Ever since, this name has been given to one or another alleged or proved erythropoietic factor

I do not believe that the observations of Carnot and Déflandre are true The increases they reported in red cell concentration in 24 hours from one injection of anemic serum are fantastically high, for example, a rise from 5 5 to 12 million in 3 days.

Confirmation of the observations of Carnot and Déflandre that were published in 1906 came in 1911 from Gibelli,[17] the increases he observed were not as large, and eight of twenty-four test animals showed no erythropoietic response In the same year, Boycott *et al.*[6] reported completely negative results The question was taken up again by Gordon and Dubin[21] in 1934, who could not observe any erythropoietic effect of the serum of animals made anemic by hemorrhage However, the logic of the idea sustained a faith that there must be something in it, that there must be a humoral mediator of erythropoiesis This faith eventually had its reward.

The contemporary state of affairs began with the experiments of Krumdieck,[36] who reported that the serum of rabbits rendered severely anemic by repeated bleeding caused a reticulosis when injected into normal rabbits, the peak occurring on the third day after injection. Neither the hemoglobin nor the red cell count rose noticeably. In 1953 reports of the experiments of Erslev and his collaborators[10-12] began to appear When these investigators injected large amounts of rabbit anemic serum into normal rabbits, 50 ml per rabbit for 2 to 4 days, there was an increase in reticulocytes in blood and normoblasts in the bone marrow. A large dose was required even for so small an effect, a 20-ml dose was ineffective. Only when 50 ml doses were given daily for several weeks was a small rise in red cell count and hematocrit observed Gordon *et al.*[24] found that filtrates of boiled plasma of rabbits rendered anemic by bleeding injected into normal rats for several weeks caused statistically significant but really very small increases in red cell count, hematocrit, and hemoglobin. The striking result was the rise in reticulocytes to a peak after 18 days of daily injections Crafts and Meineke,[8] using reticulocytosis only as a criterion, found evidence of EP in the blood of both normal and hypophysectomized rats made anemic by bleeding It was necessary to inject the anemic plasma into normal rats for 6 to 9 days.

In the last few years Jacobson and his colleagues[15, 16, 32, 33] have used increased incorporation of Fe^{59} into the blood to demonstrate an increase in EP in the blood of normal or hypophysectomized rats and in rabbits with anemia due to hemorrhage. In order to demonstrate this effect, it was necessary to increase greatly the sensitivity of the assay in rats or mice by [illegible] 95 to [illegible] ells, [illegible] hen

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Hodgson *et al.*[31] injected plasma of anemic rabbits into normal rabbits Stohlman and Brecher[54] used the sublethally irradiated rat as an animal with a depressed erythropoietic base line to demonstrate an increase in EP in rats made anemic by bleeding Their criteria were reticulocytosis and

increased uptake of Fe^{59} in the circulating blood. In 1958 Gordon *et al.* reported that normal blood perfused through the hind limb of a hypophysectomized rat stimulated hemopoiesis in the depressed marrow.[27]

To summarize the effect of hemorrhage, it is clear now that the most that can be expected from anemic serum or its boiled extract, even when injected in large doses for several weeks, is a small effect: stimulation of the bone marrow, reticulocytosis, increased uptake of Fe^{59}, or little or no actual polycythemia. We have failed to obtain a plasma filtrate able to produce a polycythemia from the severely bled horse or calf. Nevertheless, it may be considered as established that anemia caused by hemorrhage does lead to an increase in EP.

In connection with reticulocytosis as a measure of EP, I cite the use of reticulocytosis in normal mice. The material under test is injected in isotonic saline subcutaneously once a day for 3 days, the next day a reticulocyte count is made. The saline controls have averaged 2.5 to 3.0 per cent. A maximal response to a level of 8 to 10 per cent is obtained with 0.5 ml. of phenylhydrazine-anemic plasma. I do not consider a preparation positive unless it gives a maximum response if enough is used. The following is an example of what I consider an equivocal response: 1 ml. of normal horse serum gave 5.0 per cent reticulocyte count, the equivalent of 3.0 ml. gave no more.

Is the factor in the blood after hemorrhage or phlebotomy the same as or different than that in the blood after the severe anemia due to phenylhydrazine poisoning? The filtrates of boiled plasma of such blood induce a true polycythemia, as well as increased bone marrow activity, reticulocytosis, and increased uptake of Fe^{59}.[1, 22, 23, 49, 60] Until recently, in all such experiments the donor animal was the rabbit, the recipient the rat or the mouse.[34] Now Kinard and Griffin have shown that the dog may be used as donor to the rat.[35] Such boiled filtrates are active in hypophysectomized[23] and starved rats. Clearly they are not species-specific.

The polycythemia is produced, not by the serum or plasma, but by the filtrate of the plasma after it has been boiled at *p*H 5.5. Injection of the (heparinized) plasma causes a hemolytic anemia. Keighley[34] has shown that the basis for the difference is probably immunological. He found that, whereas normal as well as anemic rabbit serum is antigenic in the rat, the proteins of the boiled filtrate are not. When rats were immunized with rabbit serum and time was allowed for their hemoglobin to return to normal, they responded positively to boiled plasma filtrate. The antigenicity is not connected with erythropoietic activity.

Heating to 75° C. for a few minutes is sufficient to destroy the antigenicity of the proteins. The resulting filtrate, although it contains 20 per cent of the plasma protein, is active. The filtrate of plasma boiled for a few minutes is as active as that brought to only 75° C. Boiling for one hour may cause a loss of three-fourths of the activity, but enough remains to cause a polycythemia.

In such tests in which only 3 or 4 days are required, for example, reticulocytosis in mice or increased uptake of Fe^{59} in the blood of sensitized rats,

whole serum or plasma may be used; by these tests the plasma is 2 to 4 tim as active as the plasma filtrate made from it. We do not believe that t lower activity in the plasma filtrate is due to destruction by heat in boilin

active principle is adsorbed on the coagulated proteins, or that when t plasma (or serum) is injected not enough antibodies are formed in three four days to cause significant hemolysis. Indeed, if hemolysis did occ uptake of Fe^{59} and reticulocytes would be increased by the erythropoie stimulus of the hemolysis and this would be added to the effect of the I in the injected plasma. Hence, the total effect would not be due solely the EP injected

Jacobsen *et al* [22] have reported that their most highly active preparatic came from phenylhydrazinized animals whose livers were severely damag Phenylhydrazinized rabbits with apparently normal or very slightly damag livers gave plasma filtrates whose potency was comparable only to that bled animals, that is, that produced only a moderate reticulocytosis. Gord *et al* [22] reported that plasma filtrate from rabbits bled repeatedly for th weeks (RBC 2×10^6, reticulocytes 20 per cent) had some activity, b less than that of phenylhydrazinized rabbits

Keighley has studied this matter extensively. The best single indicati of liver damage he found is a rapid drop in temperature. Almost alwa animals with severely damaged livers had hemoglobin values below 4 0 g and their plasma was potent. However, there was a group of rabbits w hemoglobin values below 3 7 gm per cent, but with normal or only sligh damaged livers, and their plasma was potent. From animals with hig hemoglobin, the plasma was weaker or did not cause any polycythemia the usual test

The explanation may be as follows Phenylhydrazine is detoxified imm diately by the circulating red cells, which are destroyed in the process Wh as a result of this destruction, the anemia is so severe that there are r

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mice; on the second day thereafter he noted a 14 per cent increase in th red cell count. He cited such observations as confirming Carnot a Déflandre's idea of hemopoietin Like theirs, his observations have not be

confirmed, but his idea has been Some years later it was reported that the blood of animals kept at low barometric pressure accelerated the recovery of animals that had been given a standard hemorrhage [13, 43] Gordon and Dubin[21] could not confirm this They gave an injection of 10 ml of the [illegible]

not consider the result conclusive

Firm support came from the well-known experiment of Reissmann,[52] who observed polycythemia in a parabiotic rat whose partner alone had been exposed to an oxygen tension reduced gradually over 5 weeks to 8 per cent Bonsdorff and Jalavisto[2] injected the blood of anoxic rabbits into normal rabbits and observed a small increase in red cell count, but not in hemoglobin They took this as evidence of a humoral erythropoietic factor Grant[28] exposed lactating rats and mice 3 to 4 days postpartum to 300 to 400 mm Hg barometric pressure for 6 hours daily At 10 to 25 days of age the litters of these rats maintained at sea-level pressure had hematocrits, red blood cell count, hemoglobin, and oxygen capacity greater than the controls whose mothers were not exposed to low barometric pressure Grant concluded that there was a substance in the milk of the anoxic mothers that augmented eryth-[illegible]

mitotic rate than the controls Their conclusion was that low oxygen tension appeared to reduce the radiation injury by its stimulation of erythropoiesis

In an experiment that recalls the report of Jacobsen *et al*,[32] Prentice and Mirand[49] reported that they could not detect any EP in the blood of normal [illegible]

EP Carbon tetrachloride poisoning under normal oxygen tension did not have this effect It is to be noted that in their experiments, even where there was increased uptake of Fe^{59}, the hematocrit value was not increased, however, this was a short period experiment In this connection there is an [illegible]

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tive methods such as increased uptake of Fe^{59} in hypophysectomized animals or reticulocytosis, but trickled in continuously over a long period, the effect

is great enough to cause polycythemia. It is long-term effect. At first one may see increased mitotic divisions of the normoblasts in the bone marrow, but nothing in the blood for 2 to 3 days, and then only a reticulocytosis It does not seem necessary to invoke a special polycythemic factor in the later stages of hypoxia as distinguished from a hypothetical factor early in hypoxia which is capable only of mild degrees of erythropoietic stimulation However, the question is not yet settled.

Certain clinical conditions enter into a consideration of erythropoietic factors as an explanation of certain signs or as sources of erythropoietic factors Among such conditions are secondary polycythemia in clinical anoxic conditions Polycythemia vera is complicated by uncertainty regarding its etiology

Can one ascribe clinical polycythemia, primary or secondary, directly to an increase in EP? If so, the plasma or extracts should give a positive response in test animals It need not be so potent as to cause polycythemia As in the case of the hypoxia of high altitude, the concentration of EP may not be very high, but its persistence over weeks and months causes a polycythemia Bonsdorff and Jalavisto[2] reported that human plasma from cases

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The plasma in Cooley's anemia resembles that in phenylhydrazine anemia Piliero *et al* [45] found that the plasma filtrate, injected daily for five days into normal rats, produced marked stimulation of the bone marrow and a polycythemia by every criterion To have so great an effect in five days, the plasma of these cases must have had more EP than the plasma in phenylhydrazine anemic rabbits The plasma of sickle cell anemia cases gave small and variable results, but injection of urine of one case gave a small reticulocytosis and large stimulation of the bone marrow

Even in the cases of Cooley's anemia there was no correlation between intensity of the anemia or duration of the illness and the amount of EP

any increase in EP.

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formation in the bone marrow, a reticulocytosis, an increase in red cell count in the blood, but no increase in either hemoglobin or hematocrit The increase in red cell count was due chiefly to microcytes whose heightened osmotic fragility shortened their life span These workers obtained precisely the same results with the filtrate of phenylhydrazinized anemic rabbits They concluded that there are two erythropoietic factors One, most probably a lipid (I shall return to this point later), increases cell division of already existent marrow red cell precursors to produce microcytes with shortened survival time Accordingly, this factor does not increase the circulating red cell mass, nor does it accelerate the incorporation of iron

I have cited the findings of a number of workers that plasma extracts *prepared in almost exactly the same way as that of Linman et al* produced in the same test animal, the normal adult rat, a true polycythemia To account for these findings, Linman *et al* propose the existence of another erythropoietic factor that augments iron incorporation, hemoglobin synthesis and, presumably, formation of normal red cells Different chemical properties are ascribed to this factor These will be discussed below However, the only factors found thus far that affect hemoglobin synthesis directly (amino acids, iron, and glucose[4]) have no erythropoietic activity. They can be dialyzed away In the same plasma the erythropoietic material remains On the other hand, the erythropoietic factor has no direct effect on hemoglobin synthesis

In our laboratory my colleagues and I have been unable to confirm the above observations of Linman *et al* We followed their extraction procedure precisely, ether extracts of potent plasma filtrates or of the parent plasma of phenylhydrazinized rabbits were completely negative The extracts neither produced a reticulosis in normal mice, nor caused an increased uptake of Fe^{59} in starved rats The water-soluble residues were fully active Carworth-Wistar rats, the strain Linman *et al* used, gave the same full polycythemic response with potent plasma filtrate, as did Sprague-Dawley rats reticulocytosis, increased uptake of Fe^{59}, and increased red cell count, hematocrit, and hemoglobin Furthermore, we have not observed a microcytosis with weak extracts or with submaximal doses of potent extracts Rambach *et al*[49, 50] report findings identical with ours

Linman *et al* state that the anemia they induced in their donor rabbits with phenylhydrazine was below 7 gm per cent hemoglobin Keighley, who prepared the plasma filtrates we use, brings the anemia to below 4 gm per cent It seems unlikely that this is the reason for the difference in our findings One would have to conclude that in an extract sufficiently potent to produce a true polycythemia, the factor responsible for the microcytosis, which is ether-soluble, has disappeared I am unable to resolve the discrepancy

There are reports in the literature of findings that resemble those of Linman *et al*, but are not quite the same In Krumdieck's[36] experiments anemic rabbit plasma injected into normal rabbits produced a reticulocytosis, but no rise in red cell count or hemoglobin The plasma that Bonsdorff[7, 8] obtained either from anoxic patients with congestive failure or from sheep

fetuses caused a reticulocytosis with only slight and inconstant increases in hemoglobin and red cell count. Gordon[26] has reported that the serum of phenylhydrazinized anemic rats injected into normal rats gave rise to increases in reticulocytes, red cell count, hemoglobin, and nucleated red cells in the marrow, however, the boiled plasma filtrate, while it increased reticulocytes, red blood cell count, and marrow nucleated red cells, did not increase the hemoglobin All these results can be explained reasonably on the basis that the plasma was not very potent to begin with and that considerable activity was lost in boiling so that, although enough activity was left to show stimulation of the bone marrow, reticulocytosis, and increased uptake of Fe^{59}, there was not enough to produce a polycythemia.

Reports elsewhere in this monograph by Crafts, by Piliero, and by Van Dyke are to the effect that none of the known hormones, including those of the pituitary, may be considered as specific erythropoietic factors

I have referred to the evidence of an erythropoietic factor in the milk of nursing rats maintained under anoxic conditions[23] and in urine of a case of sickle cell anemia [45] Hodgson and Tohá[30, 31] reported that injection of the urine of an anemic rabbit into another rabbit after a standard hemorrhage accelerated the latter's recovery I have tested the activity of the urine of phenylhydrazinized anemic rabbits, that is, very severely anemic, on normal mice for reticulocytosis and on starved rats for increased uptake of Fe^{59}. The effects were small and variable, I am inclined to consider them, if not negative, certainly of negligible significance On the other hand, the urine of children with aplastic anemia, as reported by Van Dyke *et al*,[56] is highly potent Elsewhere in this monograph Gordon reports a similarly active urine from a case of Cooley's anemia. The plasma of these children increased the uptake of Fe^{59} in the normal test rat. In Van Dyke's case, 1 ml of urine was as active as 1 ml of plasma When a large dose was given, its erythropoietic effect was greater than the hypoxia of 15,000 feet altitude (simulated) for 14 days Such urine may very well be the best, the largest, and probably the cheapest source for preparing a highly purified erythropoietic factor. However, so far there appears to be no correlation between the clinical condition and the erythropoietic potency of the urine.[57]

Turning now to the physical and chemical properties of erythropoietic factors, there is one major area of disagreement that is restricted to those [illegible]

[illegible] pigs. Tohá *et al.*[55] reported finding in the plasma of repeatedly bled rabbits an acetone-, or chloroform-soluble factor that accelerated recovery from hemorrhage A later report from the same laboratory,[31] in which the test was increased uptake of Fe^{59}, evidently failed to confirm this finding; the plasma of bled rabbits and their acetone extracts were no more active than

the plasma of normal rabbits. On the other hand the plasma, unboiled or boiled, of phenylhydrazinized anemic rabbits was highly active. One may infer from the context that the factor was water-soluble. The most consistent and persuasive reports of a plasma erythropoietic factor that can be extracted by a lipid solvent (ether) are those of Linman *et al.*[37-40] This is the factor they observed as causing an outpouring of microcytic red cells. Linman and Bethell[37] cite observations of Sandler that batyl alcohol or the monoglycerol ether of N-octadecyl alcohol ($CH_3(CH_2)_{17}$—O—$CH_2CHOHCH_2OH$) stimulated erythropoiesis in normal and benzene poisoned rats. Elsewhere in this monograph Linman and Bethell report that daily injections of 12.5 to 25.0 mg. of synthetic batyl alcohol produced microcytosis and reticulocytosis. In their opinion it is unlikely that plasma contains so much batyl alcohol, accordingly, the effects they observed with plasma extracts can hardly be ascribed to batyl alcohol.

Hodgson *et al.* had thought that the EP factor they observed was a lipid.[20] Recently[21] they explicitly cast doubt on the ether-soluble nonprotein factor of Linman *et al.* I referred above to my inability to observe either the solubility in ether or the stimulation of microcytosis.

There is almost complete agreement on the properties of the water-soluble erythropoietic factors EP and EU. Indeed, their properties reported thus far are the same. The consensus is that these factors are considerably resistant to boiling. This was found in human cord blood,[9] anemic and cobalt rat plasma,[32, 43] phenylhydrazinized anemic rabbit plasma,[5, 22, 25, 32, 49, 59] hemorrhagic anemic rabbit plasma,[22] the plasma in human primary and secondary polycythemia,[40] and Cooley's anemia and sickle cell anemia.[45] Some activity may be lost in boiling plasma, but the loss may be more apparent than real. This point has been discussed above. Certainly, sufficient activity is retained to cause a polycythemia. The great volume of foregoing evidence outweighs two reports that the activity of potent plasma is entirely destroyed by heating to 56° C.[7] or by boiling.[10]

EP and EU are nondialyzable[5, 12, 13, 46, 59, 54, 56] and can be lyophilized,[21, 22, 60] although possibly with some loss.[22]

The activity of EP is completely lost by allowing it to stand overnight at pH 1.6 at 37° C. On the other hand, EP is quite stable at pH 4.0 and probably even at lower temperatures. It is stable at pH 10.9 for 20 hours at room temperature.

Linman and Bethell[39] discovered that EP remains in the filtrate when the filtrate of boiled plasma is precipitated with perchloric acid in the cold, this has been confirmed.[6, 59] We have found that there is some loss in activity, possibly due to the acidity. Trichloroacetic acid inactivates EP,[57] again probably because of the low pH. Slaunwhite *et al.*[53] reported some loss in activity of EP by digestion with pepsin or chymotrypsin for four hours at 37° C. We have found that the activity is completely destroyed by digestion overnight at 37° C. with either trypsin or chymotrypsin (0.1 per cent).

The foregoing properties and those described below prove that EP and EU are proteins. Boiled plasma filtrate contains 1 to 2 mg. protein per ml. The fact that EP resists boiling suggested, once its protein character was

established, that the active protein is a mucoprotein. At no time have we been able to dissociate erythropoietic activity from protein.

EP is precipitated by 80 per cent saturated ammonium sulfate; according to Rambach *et al*,[50] it is precipitated by 75 per cent saturated ammonium sulfate We have found it to be quite soluble in 70 per cent saturated ammonium sulfate EU is precipitated by ammonium sulfate[47]

Two partial purifications have been reported recently Grant *et al*[33] obtained a highly potent precipitate by fractional precipitation with 60 to 80 per cent alcohol Rambach *et al*[31] reported that the active principle can be adsorbed on the modified anion-binding cellulose DEAE, from which it is eluted by solutions of NaCl The purified preparation gave a strong sialic acid and other tests for mucoprotein Our findings agree in the main with those of Rambach *et al* However, we could not elute all the protein from DEAE We lost 90 per cent of the activity, and the active fraction was not more than 3 or 4 times as active on a protein basis as the original plasma filtrate The procedure that has given us our most active preparation is as follows The plasma is precipitated with 80 per cent saturated ammonium sulfate Water is added to the washed precipitate so that it contains 50 per cent saturated ammonium sulfate After vigorous shaking at 4° C. for some hours the suspension is filtered and the filtrate is dialyzed, made isotonic with NaCl, the *p*H adjusted to 5 5, and the filtrate is then boiled for 5 minutes. All the activity passes into the filtrate. This preparation gives a maximal response in mice with 250 μg On a protein basis it is much more active than the filtrate of boiled plasma. As Rambach *et al* found, it gives a strong sialic acid and other tests for mucoprotein, but so also do the fractions that

electrophoresis this material gave three bands, and the chief activity was found in the fastest moving band, which contained about one third of the protein

The active fraction was purified further as follows. After the ammonium sulfate was removed by dialysis, the solution was concentrated by freeze-drying to 2 per cent protein, after which saturated ammonium sulfate was added to give a 70 per cent saturation The filtrate contains the activity; it is dialyzed sulfate-free and lyophilized to dryness This material was active in all three tests employed 0 18 mg daily for 3 days increased the

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faster than α_1-globulin. There was a faint trace in the α_2-globulin area.

Rambach *et al* [30] were of the opinion that EP activity moved on paper electrophoresis with the α_2-plasma globulins In other papers in this monograph it is stated that there appears to be little difference in the mobility of the purified material used and ours

In summary, it may be said that the concept of a specific humoral erythropoietic factor has been established The factors in plasma and urine appear to be mucoprotein, whether they are the same remains to be established

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STUDIES OF RED CELL SURVIVAL*

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In vivo survival of red cells can be investigated only when some method of labeling is used † These methods may be divided into two classes. With methods of the first class, all cells are labeled regardless of age At present radioactive sodium chromate is the most useful agent in this class, but it has the disadvantage that the label is not quite permanent on the average, there is a loss of slightly less than 1 per cent daily Preferably it is used to tag a small sample *in vitro* Before injection the slight excess of uncombined chromate is removed by washing or, better, reduced by ascorbic acid to a chromic salt, in this form it does not combine with red cells The labeled cells may then be injected back into the subject from which they were taken or into another subject By injecting it intravenously, chromate also has been used to label all the cells in the circulation, although not as heavily The objection to this method is that the chromic salt to which the chromate is rapidly reduced in the blood stream labels plasma proteins and, in samples taken over the first few days, by far the greater part of the radioactivity is in the plasma Because of its relatively rapid removal, the opposite becomes true in later samples However, in the earlier samples the very much smaller red cell radioactivity that is determined by subtraction of the plasma radioactivity from that of the whole blood is liable to an unnecessarily large error Transfusion of cells labeled intravenously in this manner into a different subject is unsatisfactory because of the relatively small amount of chromium attached to each individual cell.

Also in this class are the Ashby method and its modifications, based on a natural means of identification provided by blood groups Since these

O or N and to recipients of groups A, M and, possibly, B For others, sufficiently powerful antisera are not available except for the Rh system, which should probably be avoided for other reasons Of course, cells can be followed only after transfusion If a reasonable degree of accuracy in counts is desired, the method is laborious The newer instruments for automatic counting of red cells have not been used yet to any great extent to lighten these labors

Radioactive diisopropyl fluorophosphate (DFP[32]), introduced more recently,[2] is a permanent label and, in this respect, superior to chromium, but

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† For recent discussions with extensive bibliographies see Mollison[1] or Jacobs [17]

the amount that combines irreversibly with the red cell is very small Phos phorus, a beta emitter, is more difficult to count than chromium, which give off gamma rays, and it has the further drawback of a relatively short half life While it is possible to label a sample of blood *in vitro* for transfusion (in th [illegible]

transfusion of reasonable amounts of blood is unsatisfactory because of th small amount of radioactivity transferred

Methods of the second class are those in which the cells tagged belong to a relatively restricted age group, the ideal being to confine the tag to cells of a single age N^{15}, C^{14}, and iron, both Fe^{55} and Fe^{59}, have been used for this Of these, iron is perhaps the easiest to use and, although normally there i almost complete reutilization, this can be largely suppressed[2] The long half life, especially of Fe^{55}, and the absence of excretion from the body resul in prolonged exposure to radiation, this is a barrier to the use of these iso topes, especially in man Nitrogen and carbon are less dangerous, but it i more difficult to obtain a well-defined beginning, for the uptake tends to rise somewhat slowly to its maximum

Types of Survival Curves

Disappearance of labeled red cells may be due to several agents or processe or to combinations of these, giving rise to a half dozen or more distinct form of survival curve. In every case at least some of the cells disappear becaus they have reached a limiting age.

Senescence The discovery that bile pigments originated from hemoglobin soon led to the conclusion that the life of the erythrocyte was relatively shor when compared with that of the body in which it circulated, this was con firmed in the most direct way by Ashby[5] approximately 40 years ago How ever, daily loss of red cells may be due either to incidental damage, such as the wear and tear of the circulation, or to some age limit (FIGURE 1) That the latter was the case was shown by Shemin and Rittenberg[6] who, using N^{15}, labeled a population that was relatively uniform with respect to age. The [illegible]

been confirmed many times subsequently with Fe^{59} and C^{14}, as well as with N^{15}.

[illegible]

[illegible] destruction apart from senescence [illegible]

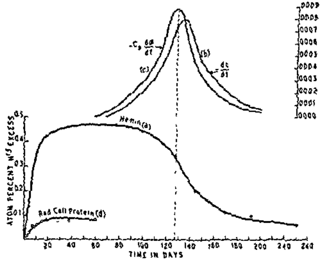


FIGURE 1 Survival of human erythrocytes of a relatively homogeneous age group labeled with N^{15}. Reproduced by permission from Shemin and Rittenberg [8]

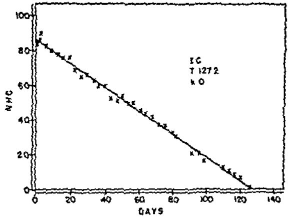


FIGURE 2 Survival of human erythrocytes transfused from healthy donors into healthy compatible recipients, followed by differential hemolysis. Reproduced by permission from Eadie and Brown [14]

The point at which this straight line cuts the time axis is the average life span It is important to distinguish this time, which is based on death from senescence only, from what may be called the survival time, that is, the time the cell actually survives without restriction as to the cause of disappearance The conception of life span thus excludes disease or accident, such as hemorrhage The reason for this apparently artificial distinction is that, when investigating disease, it is desirable to eliminate from the average deviations attributable to individual variation Life span defined in this way appears to be characteristic of the individual It is probably genetically determined — built in, so to speak—when the cell is formed in the marrow. There is no good evidence that it is affected by disease and, on analysis, statements to the contrary appear to be due to confusion with survival time as just defined, or to mistaking curvilinear disappearance for linear in short survival curves Life span varies considerably from one individual to another: normal limits are not yet known, but are likely to be as short as 100 days or less and as long as 130 or more days That is a variation of about one month on an average time of between 3 and 4 months If survival is expressed as a half life, this would mean a variation in half life of 15 days or more, in addition to the experimental errors involved in its determination The size of the deviation indicates that by this method it is possible to detect only the grosser departures from the normal

When a method of the first class is used, the equation for the survival curve is the usual equation for a straight line If cells of a single age group only are labeled (method of the second class), the curve is fitted by[7]

$$n = \frac{N_0}{1 + e^{\alpha(t-T)}} \tag{1}$$

in which the Pearl-Verhulst expression is used instead of the probability integral (FIGURE 3) The constant α corresponds, but in an inverse way, to the standard deviation the larger α, the less the spread about the mean

The cause of disappearance of cells with age is unknown Speculation would lead one to postulate either the loss of some essential constituent, perhaps an enzyme as suggested by Allison and Burn (although there is little reason to think that cholinesterase is essential to the cell), or the accumulation of some product such as immobilized protein[8] The demonstration that older cells deficient in glucose-6-phosphate dehydrogenase are more susceptible to primaquine is, perhaps, a lead[9,10]

Random loss Under this heading may be gathered all curves in which there is a constant daily loss either of cells or label unrelated to the age of the cell (FIGURE 4) Of course, loss of label is the case with chromium, here the rate of loss varies somewhat from individual to individual, and may amount to from 0.5 to 1 5 per cent daily It is usually ascribed to elution In this case the age distribution of labeled cells may be normal, if this is true, the equation derived by Sheets *et al*[11] is applicable It gives y, the number of cells present, or the radioactivity due to them on any day t in terms of the average life span T, and the fraction k lost daily, y_0 being the initial number of cells

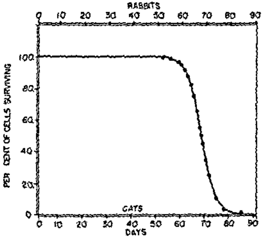


FIGURE 3 *Theoretical survival of cat and rabbit erythrocytes in the absence of random destruction when a single age population is labeled* Drawn from equation 1

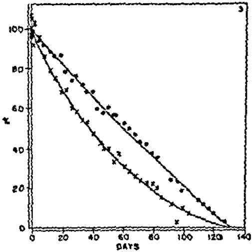


FIGURE 4 *Survival of human erythrocytes transfused from healthy donors into healthy compatible recipients, followed by* Cr^{51} *labeling (curved line) compared with differential hemolysis (straight line)* Reproduced by permission from Eadie and Brown [14]

or the radioactivity count on day zero:

$$y = y_0(1 - t/T)e^{-kt}$$ (2

This is a difficult equation with which to work, for there is no simple way of reducing it to linear form. Attempts to solve it by assuming a value for T and then solving for k by a logarithmic transformation and adjusting by a least-squares method are to be avoided as prejudicing the results, since the value of k obtained in this way depends on the value chosen for T. It is therefore arbitrary. Unbiased estimates of t and k may be arrived at by a

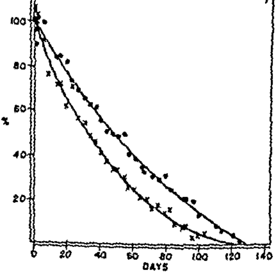


FIGURE 3. Survival of human erythrocytes from healthy donors into healthy compatible recipients. The upper curved line (differential hemolysis) shows slight random destruction; this increases the curvature of the lower line (Cr^{51} tagging). Reproduced by permission from Eadie and Brown.[14]

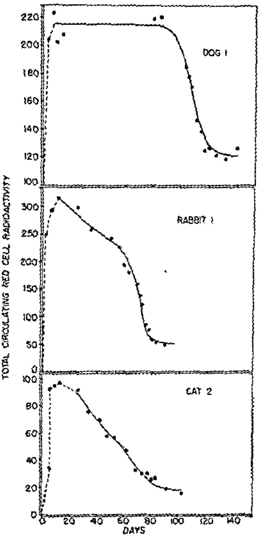


FIGURE 6 Survival of erythrocytes of a relatively homogeneous age group labeled with Fe^{59} to illustrate absence (dog) and presence (rabbit, cat) of random destruction Reproduced by permission from Brown and Eadie.[7]

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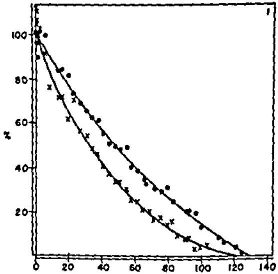


FIGURE 5 Survival of human erythrocytes from healthy donors into healthy co patible recipients The upper curved line (differential hemolysis) shows slight rand destruction this increases the curvature of the lower line (Cr^{51} tagging) Reproduc by permission from Eadie and Brown[14]

... method of fitting data to the ... approximation met

method. It is so small that it might otherwise be mistaken for a slight faster elution of the label, as in chromium experiments. Although tl

When plotted, (FIGURE 8) this differs only slightly from EQUATION 2 Here again, although differences to the eye are slight, there should be no confusion as to which equation is applicable

With methods of the second class the equation must express the presence of an exponential fall in place of the plateau This leads to

$$n = \frac{N_0(1-b)}{1+e^{(t-T)}} + N_0 b \tag{5}$$

which has been shown to fit data in a satisfactory manner [7]

Hemolytic diseases Theoretically, hemolytic disease might alter survival curves by acting on the mechanism, whatever it may be, that produces

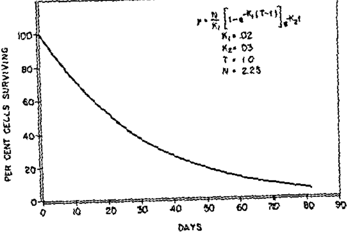


FIGURE 8 Theoretical survival of cells subjected to random destruction before and after labeling, daily loss of 1 per cent of label

senescence by destroying cells in some way unrelated to their age or by affecting the rate of cell production As previously stated, there is no evidence that the first of the effects occurs Although it belongs in the second class, destruction by antibodies is discussed more conveniently under a separate heading Other diseases of this class may be subdivided into two groups

In the first group are those diseases in which the rate of cell destruction is constant, at least over relatively long periods This happens, for example in sickle cell disease, although interrupted from time to time by crises It seems probable (FIGURES 9 and 10) that a small fraction of the abnormal cells, varying little from day to day, is exposed to very low oxygen pressures somewhere in the course of the circulation and that, probably as a result of sickling, a constant proportion, perhaps all, of these are destroyed EQUATIONS 4 and 5 apply here

The same type of steady percentile destruction is seen in animals, frequently in dogs, and almost always in cats and rabbits[7] (FIGURE 6). It has been found also in sheep It has been demonstrated not only with methods of the first class such as chromium, but also with those of the second class In the latter case the plateau in the curve between the end of labeling and the beginning of destruction from senescence is replaced by an exponential fall. The rate of removal of cells is often greater than in man and, in some instances, there is reason to believe that the loss of blood is caused by parasites in the intestines

As a consequence of these differences the equations for the survival curves given above must be modified. With methods of the first class the sample

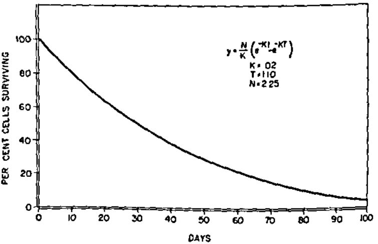


FIGURE 7 Theoretical survival of cells subjected to random destruction before and after labeling, no loss of label

of cells labeled will contain, as explained above, more younger than older cells. Making the same assumptions as before and, in addition, assuming a constant daily fractional loss, k, with a constant daily production of cells, n_0, it is easy to show that the number of labeled cells present on any day, t, after labeling will be

$$y = (n_0/k)(e^{-kt} - e^{-kT}) \tag{3}$$

When plotted, (FIGURE 7) this gives a curve not very different from that given by Janney's equation (Equation 2), in fact, it would be almost impossible to distinguish the two by inspection, although it is usually not difficult to tell which equation applies in the case under consideration This equation implies that the rate of random loss is the same before and after labeling

e chromium elution rate

rate, k_2, after labeling

$$y = (n_0/k_1)(1 - e^{-k_1(T-t)})e^{-k_2t} \tag{4}$$

When plotted, (FIGURE 8) this differs only slightly from EQUATION 2 Here again, although *differences* to the eye are slight, there should be no confusion as to which equation is applicable

With methods of the second class the equation *must* express the presence of an exponential fall in place of the plateau This leads to

$$n = \frac{N_0(1 - b)}{1 + e^{(t-T)}} + N_0 b \tag{5}$$

which has been shown to fit data in a satisfactory manner [7]

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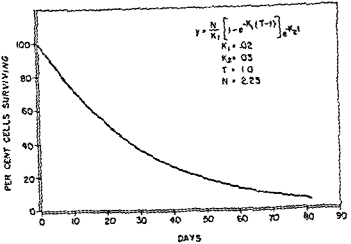


FIGURE 8 Theoretical survival of cells subjected to random destruction before and after labeling, daily loss of 1 *per cent* of label

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The second group consists of those diseases in which the
irregular and, with these diseases
these cases the age
differs from norn
It may be noted who menstruate a
unsuitable as dor many experiments
As yet, no analysis has been made of the effects of changing rates of erythr
poiesis on the survival curve.

Storage The damage produced by storage is apparently all or none, th
is, stored cells either survive normally on transfusion or do not surviv

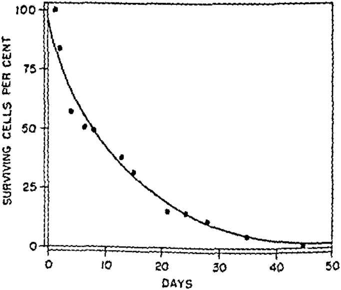


FIGURE 9 Survival of sickle cell erythrocytes in a normal recipient, showing high r of random destruction Experimental points from Singer and Fisher [14]

beyond the first day or so The percentage surviving depends on the soluti
used for preservation and for prevention of coagulation, the temperature
storage and, of course, the time of storage The survival curve shows a rap
drop as the nonviable cells are being eliminated The duration of this
usually less than 24 hours, but may be slightly longer when
The drop is followed by a normal surviv
viable cells have the same
show
t
l rage the span of these ce
v after the storage, so that it is quite cle
t of normal senescence was decreased to the vanishing po
during storage However, if senescence were due to a chemical process th
for example, doubles in rate for every rise of 10° in temperature, then t

extent of senescence would be less than 1 one-thousandth of that at body temperature. For a storage period of 10 months this would be 1 one-hundredth of a month, or less than a day, and this is not detectable

From a practical point of view interest centers on the extent of the initial fall, for the optimum storage conditions are those in which the number of nonviable cells is least As a measure of these, earlier workers used the percentage survival two or three days after transfusion It now seems simpler

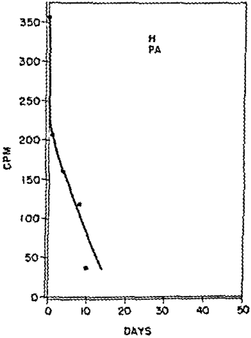


FIGURE 10 Survival of erythrocytes in a patient with pernicious anemia Intravenous Cr^{51} labeling Unpublished data by courtesy of Wightman and Hazlett, University of Toronto, Toronto, Canada

to plot the survival curve on semilog paper, in this case the points fall very close to a straight line for about the first three weeks A least-squares extrapolation of the line back to the day of transfusion probably gives the most accurate measure of the number of viable cells The number of cells

with P^{32}

Incompatibility and antibody formation. Pioneer work in this field has been done on this side of the Atlantic Ocean by Swisher and Young[14] and,

on the other side, by Mollison[1] and others, but much remains unknown Most of the work on changing antibody titer has been done at what mus be regarded for our purposes as very high titers, that is, those that are hig enough to produce agglutination or hemolysis in a few minutes *in vitro* afte dilution In the case of survival curves we must consider cases in which th highest antibody titer is just sufficient, undiluted, to cause the disappearanc of erythrocytes *in vivo*

At least three different survival curves are found when small volumes c incompatible or potentially incompatible red cells are transfused (FIGURE 12 The first type of curve is seen when the recipient's blood contains a hig titer of antibody before transfusion. Incompatible cells are destroyed in

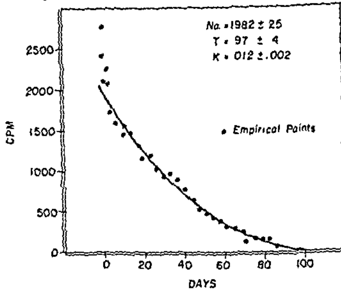


FIGURE 11 Survival of erythrocytes stored in glycerol at $-70°$ C. for 1 year, lab with Cr^{51}

few minutes The rate of elimination is said to be exponential, but th retical reasons why this should be so are not yet forthcoming.

The second and third types are seen when no antibody is present at time of transfusion if the recipient is capable of producing an antibody these cells The difference between the two is in the rate of antibody forr tion, which is fast in the second type and slow in the third. The reason making this distinction is the very different shape of the two curves. It reasonable assumption that the resistance (or susceptibility) of the eryth cyte to antibody is not quite uniform, but varies from cell to cell, being tributed in the usual "normal" fashion. After transfusion of these

rising, but has

Throughout

, possibly, also

random destruction. The antibody titer then reaches a level at which

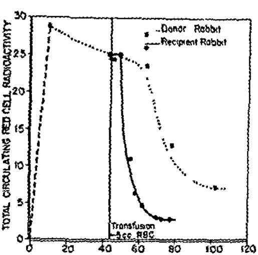


FIGURE 12 Survival of transfused cat erythrocytes, showing the effect of rapid isosensitization, reproduced by permission from Brown and Eadie [7]



FIGURE 13 Theoretical survival curves, showing different rates of antibody development

more susceptible cells are affected and, as it increases, more and more cells disappear from the circulation and the survival curve shows an S-shaped drop The equation for such a survival curve, based on these assumptions, is

$$y = \tfrac{1}{2}y_0(1 - t/T)e^{-kt}\left[1 + \tanh\frac{\delta}{2}(T - \tau)\right] \qquad (6)$$

The constant b is related to the rate of antibody formation and τ is the time when the antibody titer has risen to such a point that half the injected cells are destroyed. Antibody formation will be characterized quantitatively by two numbers, one being the rate of formation, and the other the amount necessary to affect half the cells. If the rate is slow, the appearance of the

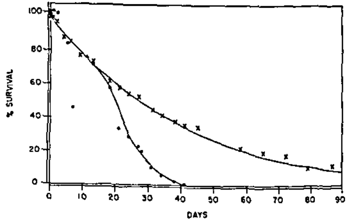


FIGURE 14. Survival of human cells from a pump-oxygenator with control

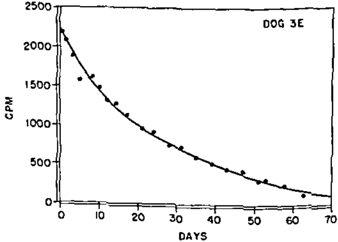


FIGURE 15. Survival of transfused dog cells, labeled with Cr^{51}

curve alters (type 3). This type has often been seen in dogs, but is apparently less common in man.

It must be emphasized (FIGURES 13 to 17) that there is nothing in this curve itself to indicate that the toxic agent is an antibody, and it is entirely [illegible] the

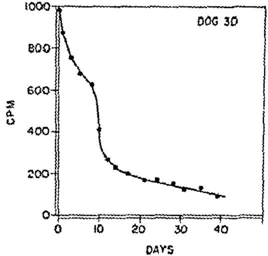


Figure 16 Survival of transfused dog cells labeled with Cr^{51}

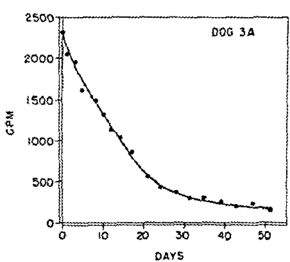


Figure 17 Survival of transfused dog cells labeled with Cr^{51}

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CELLULAR KINETICS AND IRON UTILIZATION IN BONE MARROW AS OBSERVED BY Fe^{59} RADIOAUTOGRAPHY*

Edward L. Alpen and Doris Cranmore

United States Naval Radiological Defense Laboratory, San Francisco, Calif

Since the radioactive isotopes of iron have been available, a great deal of effort has gone into studying the pathways of iron metabolism and the kinetic interrelationships of cellular production and utilization of iron. Several techniques have been proposed for the evaluation of erythropoietic function using, for example, the plasma iron turnover time,[1] rate of appearance of labeled red cells,[2] and the fraction of the injected tracer that eventually appears in peripheral blood.[3] All of these have proved to be useful tools, but their application has suffered from incomplete knowledge as to the reactions occurring in the bone marrow cells and leading to the observed end points. Two groups have had some success in the technique of radioautography for evaluating iron incorporation at the cytological level.[4,5] However, their observations were exploratory and have not been extended subsequently. It is the purpose of this study to develop further the technique as a means of investigating the relationships between cellular development and incorporation of the radioisotope.

Methods

Male mongrel dogs of 7 to 10 kg. body weight were used after several weeks of observation and treatment in the laboratory's kennels. All animals were immunized against rabies, distemper, and canine hepatitis. At the time of experiment the animals were injected intravenously with 0.6 to 0.8 mc. of $Fe^{59}Cl_3$ buffered with sodium citrate. Plasma and blood samples were drawn from the external jugular vein, and marrow was sampled by aspiration from the ribs with the Jones sternum needle. The marrow was collected into tared, heparinized, autologous plasma and the dilution of the drawn marrow calculated from the sample weight and plasma weight. After collection of the marrow sample, cells were dispersed by forcing the sample through a fine-mesh nylon screen.

A portion of the marrow aspirate as well as marrow "plasma" was plated on 1-in. cupped planchets for radioactivity measurement. Peripheral blood and plasma were also plated for radioactivity measurement. Counting was done with an end-on NaI(Th) crystal and photomultiplier. Standard samples of the injected isotope were prepared and counted simultaneously for isotope-decay correction and for calculation of the injected radioactivity.

Hematocrits and erythrocyte counts were done on blood specimens. Hematocrit, erythrocyte counts, and nucleated cell counts were done on marrow specimens. All these were done in duplicate by standard clinical

* The opinions or assertions contained herein are our own and are not to be construed as official or reflecting the views of the Navy Department or the Naval Establishment at large.

laboratory procedures. Differential nucleated cell counts of marrow were made on Wright-stained cover-slip smears. All cells were classified as a check on the homogeneity of marrow-sampling techniques. However, only erythroid classifications are germane to this problem These cells were classified as erythroblast (rubriblast), pronormoblast (prorubricyte, rubricyte), and normoblast (metarubricyte).

For radioautographs, thin slide smears were made of each blood sample and of the diluted marrow specimens. In addition, a portion of each marrow and blood specimen was supravitally stained for reticulocytes with brilliant cresyl blue, and thin slide smears were made of these stained preparations All smears for radioautographs were fixed in absolute methyl alcohol and air-dried They were then surmounted with NTB-1 permeable base stripping

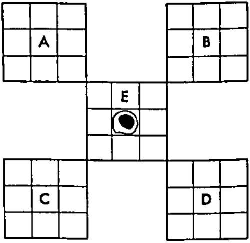


FIGURE 1 Grain-counting grid used in estimating cellular radioactivity Cellular grain count is the total number of grains in square E minus the average of squares A, B, C, and D The area of square E is such that the maximum range of any β track is included

film* by flotation in a water bath at 22° C. The air-dried slides were stored over desiccant in air- and light-tight cans at −10° C for 2 to 3 weeks

The exposed film-mounted slides were developed in Eastman D-19 at 22° C. for 7 min and cleared in Eastman acid fixer-hardener for 10 min

A quantitative estimate of the Fe^{59} activity per cell was obtained by grain counting With an ocular graticule, a square was delineated that was, in area, at least ninefold larger than the projected area of the cells The grains were counted with the cell in question centered in this square The background was determined as the average number of grains in four equal-sized adjacent areas FIGURE 1 pictorially describes the counting arrangement used Chosen at random, fifty cells of each nucleated cell type and reticulocytes were counted for quantitative estimation of radioactivity In the case of erythrocytes, grain counts were done only over those cells that were subjectively chosen as being positively labeled

Results

TABLE 1 shows the plasma iron "half times," the maximum peripheral incorporation of radioiron, and the time for half of this peripheral label to

TABLE 1
RADIOACTIVITY DATA ON BLED AND CONTROL DOGS

Measure	Control (5)*	Bled (3)* 40 ml kg	Bled (1)* 100 ml kg
Plasma clearance, $t_{1/2}$, min	98 ± 31	39 ± 2	26
Maximum incorporation percentages injected dose	55 ± 8	89 ± 18	91
Time for one half maximum uptake (days)	2 8 ± 0 6	2 4 ± 0 3	1 6

* Number of subjects

make its appearance The usual finding of accelerated plasma clearance and increased incorporation efficiency is seen in animals that had been bled to a total of either 40 ml/kg or 100 ml kg during the week just preceding injection of the isotope

In FIGURE 2 it may be seen that, by the technique of subjective examination of cells, almost all nucleated marrow cells of the erythroid series are labeled within the first 4 to 8 hours after injection of the isotope In fact, in the control animals only a few cells of the erythroblast group remain unlabeled after 4 to 6 hours, and this small proportion of unlabeled cells is most probably attributable to failure to classify correctly a small number of early blast type cells that are truly myeloid in their eventual development No significant differences between bled and control animals are seen in the rate of labeling, but in all bled dogs, as in the example shown, there is a failure to reach the stage of complete labeling characteristic of the control group In addition, the plateau of maximum labeling seen in the controls is observed only through the twelve-hour point in the anemic individuals Some of these differences may be explained by the observed fact that the level of labeling is somewhat lower in the anemics, most probably because of the competition for tracer by the larger cell population This increases the probability of classifying a cell as unlabeled. Alternatively, the explanation might be put forward

that labeling continues from a secondary pool, turning over more slowly [illegible] where cellular [illegible] evidence [illegible] remains slightly radioactive in the controls for some time after the initial rapid disappearance phase and therefore could be reflecting movement of iron from a labile nonplasma pool to marrow. It should be mentioned as bearing on

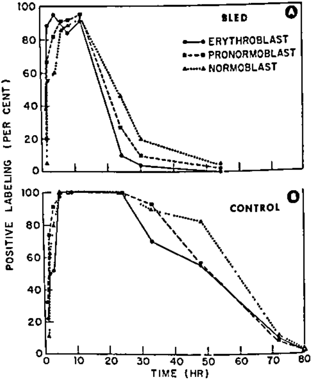


FIGURE 2 Fraction of nucleated marrow cells labeled, as adjudged by subjecti[ve] examination of autoradiographs

this problem that, as early as twenty-four hours after injection of the lab[el] significant numbers of reticuloendothelial cells showed positive radioaut[o]graphs These cells contained identifiable remnants of nucleated erythro[id] cells and erythrocytes It is entirely possible that part of the continui[ng] labeling observed may result from destruction of a certain small number [of] labeled cells. In no case were cells other than erythroid or reticuloend[o]thelial labeled

A significant difference between our *in vivo* results and the *in vitro* resu[lts] of Lajtha and Suit[6] should be mentioned The latter reported that on[ly] 10 to 50 per cent of the late polychromatic and orthochromatic normoblas[ts]

became labeled, whereas we observe 100 per cent labeling of this group which, in our nomenclature, comprises most of the normoblast category. They also reported that basophilic normoblasts (our pronormoblast) reached a stage of labeling equal to that of the pronormoblast (our erythroblast). FIGURE 3 shows our data on cellular radioactivity. Clearly, in both bled and control dogs the erythroblasts generally are labeled to a level nearly twice as high as that of the pronormoblasts. However, after 24 hours, the median cellular activities are the same for all groups. Of the discrepancies between our

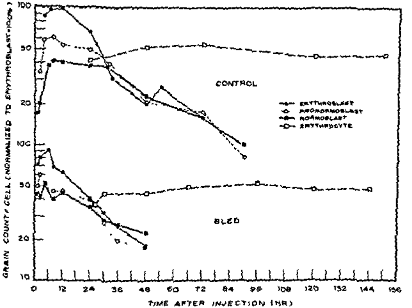


FIGURE 3. Cellular radioactivity of individual marrow cells. The plotted values are medians of the net radioactivity of fifty cells of each type. The medians are then normalized to the erythroblast value to correct for variations in exposure time and injected activity.

data and those of Lajtha and Suit, the most easily explained is that of the fraction labeled. Because the general level of labeling in their study is lower there would be more tendency to reject cells as unlabeled. However, no such explanation is forthcoming for the differences in levels of labeling.

The slopes of the lines describing the loss of activity are generally equal in either the bled or control dogs. However, because we erroneously anticipated a more rapid fall of these curves for the anemics, there is no point later than 48-hour postinjection. Also in FIGURE 3 are the grain counts on erythrocytes that appear in peripheral blood.

From application of the technique first described by Suit[3] it is possible to determine the total cellularity of bone marrow and, from this estimated value

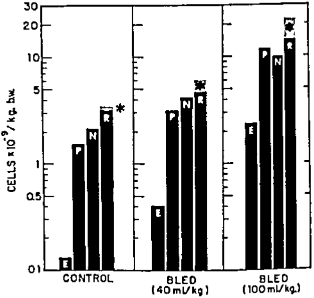


FIGURE 4 Total erythroid cellularity in the bone marrow E, P, N, and R refer respec tively to erythroblast, pronormoblast, normoblast, and reticulocyte The asterisked po tion of the reticulocyte column represents the peripheral blood component.

and the differential nucleated cell counts, to establish the numbers of th various erythroid precursors The calculated values for the animals studie are shown in FIGURE 4

Discussion

At the outset, the primary aim of this work was to describe the happenin in the marrow compartment during the transfer of iron from plasma to matu erythrocyte Certain facets of this process are immediately apparent fro the data shown Incorporation of the radioactive tracer is a rapid proce occurring in all nucleated cells of the erythroid series. From the time

characteristics such as complete and uniform mixing of the label, but mu

on the other must represent essentially a frequency distribution of maturation times or "staying times" in the nucleated portion of the erythron for all those cells that were labeled, as well as for their generated offspring. As a corollary to this statement, the time at which one half of the total cell number has appeared in peripheral blood is an estimate of a mean cell maturation time or, possibly, better described as the mean duration of stay for a cell in the nucleated portion of the erythron. It is interesting to observe that the

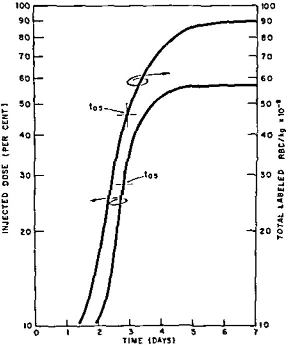


FIGURE 5 Appearance of radioactivity and of labeled erythrocytes in peripheral blood following injection of Fe^{59}. The curves are referable to the ordinate indicated by the arrows. Time for half maximum is indicated as $t_{0.5}$.

value calculated from the curve describing the appearance of labeled cells is essentially identical with that calculated from the usual radioactive iron utilization curve. TABLE 2 compares these values for the bled and for the

peripheral blood (FIGURE 5)

A well-known relationship of cellular dynamics is that the turnover time of a cellular compartment is equal to the ratio of mitotic duration to mitotic index. As the mean maturation time calculated above is also presumably a

turnover time for the nucleated erythron, it should be possible to compare these two derived measures independently. The average mitotic index for the control dogs was determined to be 0.035 on 18,000 cells observed. If the mitotic duration is assumed to be 0.5 hours, the turnover time from this calculation is $14\frac{1}{2}$ hours. If, on the other hand, the mitotic duration is assigned a value of 1 hour, the calculated turnover time is still only 29 hours, far short of the observed value of 70 hours derived from the red cell appearance curve.

However, there has been no correction made to the mitotic index calculation for mean time spent in the bone-marrow reticulocyte stage, which we shall show to be of the order of 26 hours. The total turnover time for a 1-hour mitotic duration then becomes 29 + 26, or 55 hours. The discrepancy most probably lies in the calculation based upon mitotic index, as this method

TABLE 2
MEAN DURATION TIME OF CELLS IN THE NUCLEATED ERYTHRON

	From uptake curve (days)	From labeled RBC curve (days)
Control	2.8	2.9
Bled 40 ml./kg.	2.1	1.8
Bled 100 ml./kg.	1.2	1.3

assumes that no cells from other compartments are entering the one in question. Kindred,[9] using this same technique in a very careful study of bone-marrow cellular dynamics, pointed out that he could not account for all the erythroid cells produced by division within the identifiable erythroid compartment. The discrepancy observed by him and here seen again must be accounted for on the basis of the number of hemocytoblasts differentiating into the erythroid series. The technique of estimating turnover time from [illegible] um [illegible] ally

produced. From the ratio of the logarithms of the number of nucleated cells labeled at the time of injection to the number of labeled red cells, an estimate of the number of divisions that occur during the maturation of the labeled nucleated erythron may be obtained. These data are presented in TABLE 3.

It is interesting to observe that, even though the number of cells produced varies by almost tenfold in these animals, the ratios are essentially constant [illegible] b [illegible] ro [illegible] on [illegible] that can have occurred can be determined from the ratio, again logarithmically, of the maximum erythroblast grain count to the minimum grain

count observed in the labeled erythrocytes This maximum value is 2 3 divisions for control animals, 2 2 divisions for 40 ml /kg bled animals, and 2 4 divisions for the single 100 ml /kg bled dog

All the data considered in the foregoing analysis point up the problems involved in interpreting the kinetics of utilization of radioactive iron None of the measures that have been suggested as techniques for estimating marrow erythropoietic activity is sensitive to the tremendous range of responsiveness of which the marrow is capable Maximum iron incorporation reflects only a 60 per cent increase in the face of fivefold increase in numbers of

TABLE 3
RELATIONSHIPS BETWEEN LABELED NUCLEATED CELLS AND MATURE ERYTHROCYTES PRODUCED

Erythroid cells (calculated) per kg	Labeled RBC/kg	Number of divisions
Controls		
3 2 × 10⁹	8 8 × 10⁹	1 5
4 9	10 1	1 1
3 1	6 5	1 1
3 9	11 1	1 5
		Mean 1 3
Bled 40 ml /kg		
6 9 × 10⁹	12 0 × 10⁹	0 8
7 9	17 0	1 2
8 7	23 0	1 4
		Mean 1 1
Bled 100 ml /kg		
24 0 × 10⁹	58 0 × 10⁹	1 3

erythrocytes produced in a test period Qualitatively, the same statements are also true for plasma clearance and the time for half maximum uptake Part of the reasons underlying the difficulties is that in accelerated production with increased cellularity the amount of iron incorporated in each cell is, perforce, decreased At the same time, normal utilization is sufficiently high to prevent wide excursions in amounts incorporated The fact that the cellular dynamics of the expanded marrow compartment are not altered seriously and that increased output is accomplished essentially by normal maturation processes from an enlarged pool also leads to insensitivity of the various methods

Observations on cellular life spans In pursuing the basic course of this investigation, other incidental data that are contributory to understanding the cellular dynamics of the erythron have become available. If one considers the curve for decrease in cellular radioactivity of a single cell species,

such as the erythroblast, as plotted in FIGURE 3, it is apparent that reduction of the median or mean value for cell grain count can occur only through division of the cell, nonrandom loss of cells from the labeled population, or entry of large numbers of unlabeled cells. By rejecting all unlabeled cells from the population analyzed and using the median of the remaining group to reduce effects from nonrandom removal of cells, it is possible to estimate the mean time required for cell division. The technique is applicable only to the erythroblasts, as they are not being affected by new labeled cells entering the population. From these curves an estimated mean time for cell division of 21 hours for both control and bled dogs is derived. In the one

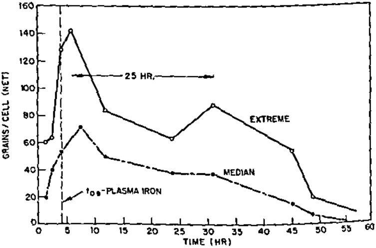


FIGURE 6. Median and extreme grain counts of pronormoblasts in dog Cu (control dog). $t_{0.9}$ indicates the time at which 90 per cent of the circulating radioiron has disappeared from plasma.

severely bled dog this value is 13 hours, but further studies are required to verify this observation. Independently, the same parameter may be estimated as in the example in FIGURE 6. If it is assumed that when the cell divides it very shortly thereafter becomes identified with the next most developed type in the series, we may arrive at estimates of division times for a cell type by their arrival in the next compartment. The cells cannot be readily identified using a population estimate such as the median, because the infinite number of out-of-phase generations masks all effects arising from division. However, the extreme values clearly show the data desired. The peak seen in FIGURE 6 with grain count of 140 probably resulted from th[illegible] were presen[illegible] epresents th[illegible] ɔtained there[illegible] e shown an[illegible] ranges from 20 to 26 hours for all animals, bled or control.

From the grain counts of the large number of reticulocytes in the marrow sample another series of estimates may be obtained but in this case they are probably less related to division and represent merely "arrival times" from various predecessor labeled groups. A very early peak at 2 hours (FIGURE 7) represents direct reticulocyte labeling. The peak at 4 hours is the arrival of the first polychromatophilic or orthochromatophilic normoblasts, probably without division. The third peak represents arrival of the pronormoblast, while the last peak most probably is the appearance of a few erythroblasts that have undergone no more than one division in their transit. Note the agreement between this value of 72 hours for transit or turnover time and the value of 70 hours obtained from the peripheral appearance curve for erythrocytes. *Lajtha and Suit[6] suggested that the reticulocytes arise directly*

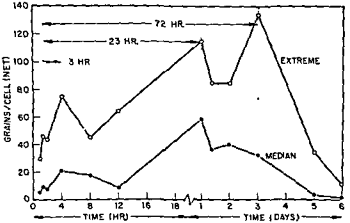


FIGURE 7. Median and extreme grain counts of marrow reticulocytes on dog BI (control dog)

from "early polychromatophilic normoblasts" rather than from the later more orthochromatic cells. The data shown in FIGURE 7 clearly indicate that this is not the case, as reticulocytes with grain counts quite similar to those seen in the latest of the orthochromatic normoblasts make their appearance early. There is no reason to believe that the reticulocyte is not a normal stage of maturation of all erythrocytes, and good evidence in support of this position can be obtained by comparing estimates of reticulocyte life span by several techniques. One method makes use of the simple, well-known approach that the time a cell spends in a given stage of development is in direct proportion to the size of its compartment and the size and mean stay time in succeeding stages. From an estimated mean cell life of 105 days and the known red cell volume of the dogs used, along with the total size of the marrow reticulocyte compartment (FIGURE 4) a mean stay time for the reticulocyte in bone marrow is calculated to be 24 hours. Of course, this calculation can be made only in the control dogs where steady-state condi-

tions exist In FIGURE 8 the grain counts for marrow reticulocytes are classified by intensity of reticulum staining The minimum stay time for the heavily reticulated cell that makes up the preponderance of marrow reticulocytes is marked on the figure as 22 hours. A maximum if less probable value of 30 hours may be assigned by reckoning from time zero. The range then is 22 to 30 hours, and the most probable value is 24 to 26 hours. Lajtha[19] has quoted a value of 24 hours for bone marrow reticulocyte life span. We have not computed life spans for peripheral reticulocytes. If any reasonable proportion of the nucleated cells matured without entering the reticulocyte

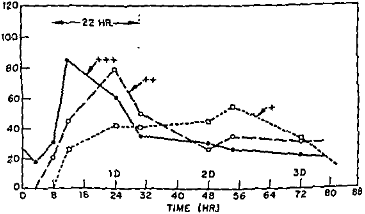


FIGURE 8 Median cellular radioactivity of marrow reticulocytes on dog BI (control dog) when cells are first classified by intensity of reticulum straining Note that approximately four fifths of marrow reticulocytes fall in category 3 plus

stage, the first computed value would tend to underestimate seriously the time of the reticulocyte life span.

Summary

A method is described for analysis of the processes of iron incorporation in erythrocyte precursors. It has been shown that all nucleated cells of the erythron incorporate iron, but that the quantity of tracer incorporated and therefore the rate of total iron incorporation of the cell is inversely proportional to the state of differentiation or development of the cell The intracellular iron is the only apparent intermediate between plasma iron and hemoglobin iron, but some evidence is presented that indicates a small reservoir of less rapidly turning over iron. The appearance of radioactivity in

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erythroblast and the pronormoblast each are approximately 21 hours in either bled or control dogs A limit can be set upon the maximum divisions occurring from entry into the erythropoietic series to completion of the development of the erythrocyte This value is 2 3 for either bled or control dogs Absolute values can be assigned to an "average" number of divisions occurring during development from knowledge of the ratio of the total number of nucleated cells labeled to the total number of erythrocytes produced This value is 1 3 in control or bled dogs Evidence is also presented to show that the reticulocyte is a normal stage of development for all erythrocytes

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PATTERNS OF NEUTROPHILIC LEUKOCYTE DEVELOPMENT AND DISTRIBUTION*

Harvey M. Patt and Mary A. Maloney

Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Ill

The neutrophil life span is an essential parameter for evaluation of neutro phil balance and its control In the physiological steady state, the distribu tion of neutrophils in marrow, blood, and periphery is directly proportiona to the time spent in the various tissues Estimates of the sojourn of neutro phils in peripheral blood vary from hours to days, depending upon the experi mental procedure, this uncertainty raises important questions about th peripheral distribution of neutrophils and its contribution to the regulatio of the level of circulating cells Several lines of evidence—for example, fron

nevertheless, there is some reason to think that neutrophils may ordinari move back and forth between blood and extramedullary sites This follow from consideration of the number of neutrophils in marrow and blood i relation to the time spent in these sites, as estimated by endogenous labeling with P^{32} The requirement for a blood-tissue exchange may receive suppo in another way, since the apparent turnover time of circulating neutrophil based upon estimates of the rate of production is greater than that derive from the disappearance of exogenously tagged cells.[8] However, this aspe of the problem of neutrophilic balance remains something of an enigm which can be resolved only by accurate appraisal of the neutrophil's life cycl

This paper is concerned with preliminary studies of the neutrophil's li cycle, as revealed by radioautographic analyses of marrow and blood fro dogs receiving tritiated thymidine. A fairly precise localization may b achieved with the labeled nucleoside because of its specific incorporatio into desoxyribonucleic acid (DNA) and the short range of the tritium bet particles Since neutrophilic leukocytes are part of a renewal system, th

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dosage of 100 μc per kg of body weight The experimental details hav been reported elsewhere [9, 10]

Radioautographs of the neutrophil series are shown in FIGURE 1 I almost all of the radioautographs, 4 grains over a nuclear area represented

* The work reported in this paper was performed under the auspices of the United Stat Atomic Energy Commission, Washington, D C

statistically significant increase above background ($p < 0.01$). About 15 to 20 per cent of myelocytes and erythroblasts are labeled within 2 hours after injection of the tritiated thymidine in the dosage employed. Since the precursor is available only for a brief time and labeled telophases are not seen during this interval, it would appear that the period of thymidine incorporation occupies at least 15 to 20 per cent of the cell generation time. However, the latter is probably an underestimate, since there is reason to believe that more cells would be labeled with a higher dosage of the precursor.[11] Moreover, our recent studies suggest that a substantial number of myelocytes are in a transitional stage between the last mitosis and emergence as metamyelocytes and, therefore, would not participate in DNA synthesis.

The tritium label appears first in the immature cells, which ordinarily contribute to renewal of the marrow population by mitosis. The distribution

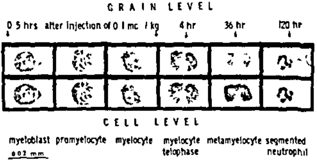


FIGURE 1. Myeloid cells labeled with tritiated thymidine. Segmented neutrophil from peripheral blood; other cells from marrow.

of labeled cells then progresses in an orderly manner from the less to the more differentiated forms. Since approximately half of the labeled DNA remains in the daughter cells of a labeled progenitor, several mitotic cycles may be required before the label is no longer detectable above the background. Densely labeled telophases are found in the marrow by 4 hours. This is followed by a rapid increase in the number of positive myeloid cells with time as shown in FIGURE 2. The number of positive cells is expressed in terms of a normalized distribution of the various cell types for this comparison of successive samples. The mean turnover time of the proliferating myeloid elements may be approximated from the increase and distribution of labeled cells. This is about 15 hours, which is in agreement with inferences based on mitotic counts,[7] but less than estimates derived from radioautographic studies of marrow cultures.[12] The number of positive cells begins to decrease by 48 hours, due chiefly to the dilution of the label by further mitosis. Eventual consideration of the temporal relationship between thymidine injection and the increment in labeled mitoses, as well as the decrement in grain counts, should provide a more complete picture of the life cycle of the

PATTERNS OF NEUTROPHILIC LEUKOCYTE DEVELOPMENT AND DISTRIBUTION*

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The neutrophil life span is an essential parameter for evaluation of neutrophil balance and its control. In the physiological steady state, the distribution of neutrophils in marrow, blood, and periphery is directly proportional to the time spent in the various tissues. Estimates of the sojourn of neutrophils in peripheral blood vary from hours to days, depending upon the experimental procedure; this uncertainty raises important questions about the peripheral distribution of neutrophils and its contribution to the regulation of the level of circulating cells. Several lines of evidence—for example, from [illegible] injection of leukocyte

[illegible]

with a large reservoir. Although recovery from abruptly induced neutropenias seems to be accomplished primarily by release of cells from marrow, nevertheless, there is some reason to think that neutrophils may ordinarily move back and forth between blood and extramedullary sites. This follows from consideration of the number of neutrophils in marrow and blood in relation to the time spent in these sites, as estimated by endogenous labeling[6-8] with P^{32}. The requirement for a blood-tissue exchange may receive support in another way, since the apparent turnover time of circulating neutrophils based upon estimates of the rate of production is greater than that derived from the disappearance of exogenously tagged cells.[8] However, this aspect of the problem of neutrophilic balance remains something of an enigma, which can be resolved only by accurate appraisal of the neutrophil's life cycle.

This paper is concerned with preliminary studies of the neutrophil's life cycle, as revealed by radioautographic analyses of marrow and blood from dogs receiving tritiated thymidine. A fairly precise localization may be achieved with the labeled nucleoside because of its specific incorporation into desoxyribonucleic acid (DNA) and the short range of the tritium beta particles. Since neutrophilic leukocytes are part of a renewal system, the kinetics of their development *in vivo* can be determined from temporal changes in the labeling of the various cells in the developmental sequence. In order to minimize departures from the steady state, these studies were performed on trained beagles with marrow samples aspirated under local anesthesia. Tritiated thymidine (360 to 390 mc./mmole) was given intravenously in a dosage of 100 μc. per kg. of body weight. The experimental details have been reported elsewhere.[9, 10]

Radioautographs of the neutrophil series are shown in FIGURE 1. In almost all of the radioautographs, 4 grains over a nuclear area represented a

* The work reported in this paper was performed under the auspices of the United States Atomic Energy Commission, Washington, D. C.

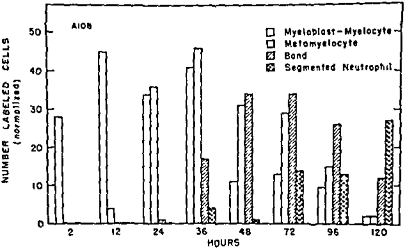


FIGURE 3 Temporal distribution of labeled myeloid cells in marrow

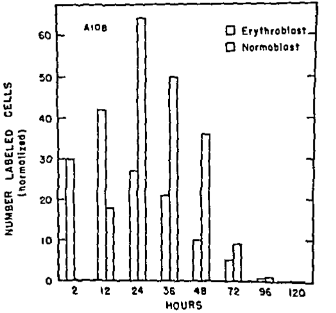


FIGURE 4 Temporal distribution of labeled nucleated erythrocytes in marrow.

various proliferating cells.* Since myeloblasts and promyelocytes compris only a small percentage of the total, these cells have been included with th myelocytes for preliminary analysis of each marrow sample.

The pattern of neutrophil development in a dog is depicted in FIGURE 3 There is a comparable progression of the label in nucleated erythrocyte (FIGURE 4), only a small percentage of lymphocytes is labeled in the marrow samples from 12 hours to 5 days after thymidine injection. It will be note from the data in FIGURE 3 that labeled metamyelocytes appear in the marrow

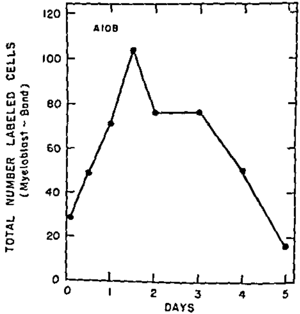


FIGURE 2 Number of labeled myeloid cells in marrow after injection of tritiated thymidine

blood by 3 to 4 days The peak of labeled cells in the circulation (25 to 30 per cent) is at [illegible]
phils in marrow [illegible]
bered that there [illegible]

* Such studies are being made and point to a mean generation time of 16 to 18 hours for myelocytes and about 9 hours for myeloblasts and promyelocytes

is consistent with the finding that labeled myelocytes are detectable in marrow for several days after injection of tritiated thymidine, though with decreasing grain concentration. It will be recalled that the peripheral half time of 12 to 24 hours corresponds to the 15-hour mean generation time approximated from the rate of increase of labeled cells in marrow.*

Information concerning the normal pathways for neutrophile utilization and removal is incomplete. Many neutrophils are apparently destroyed in discharging their functions of removing cellular debris and helping to maintain the sterility of the internal environment. Probably large numbers of cells are also lost in various secretions and excretions. Thus, it is reasonable to suppose that the peripheral time span of neutrophils may be less dependent upon age than that of erythrocytes. The data presented here are suggestive of such a random utilization. Departures from an exponential disappearance in situations where all labeled cells contribute to the analysis could be attributed to continuing release of labeled cells after the peak is reached. As noted by us previously,[7,8] the heterogeneity of the labeled proliferating population could exaggerate the peripheral time span estimated from the rise and fall of the blood level of DNA-P^{32} after labeled cells begin to appear. Since the more homogeneous classes disappear with one half time of 12 to 24 hours, the peripheral pool of neutrophils may not be nearly as large as has been inferred. Perhaps only 5 to 10 times as many cells may reside in the periphery as in obvious circulation, in contrast to previous estimates of some 50 times.[4,5] A substantial part of this reservoir may exist within the confines of the vascular system, possibly to a considerable extent as marginal cells. These results also suggest that there may be 3 to 4 times as many developing neutrophils and a nearly equivalent number of segmented neutrophils in marrow as in the periphery (vascular and extravascular). The fact, noted earlier, that recovery from acute neutropenia is attributable in large part to release of cells from marrow lends credence to the present estimates of neutrophile balance.

In conclusion, it should be emphasized that the data presented for neutrophile development and distribution in the dog are provisional and are intended primarily to illustrate the applicability of the method for evaluating the problem of neutrophile balance. More detailed analyses will be necessary to establish the life cycle of the various proliferating elements and the chronology of maturation in marrow and disappearance from the periphery.

Acknowledgment

We thank Eugenia Jackson for technical assistance and Alice S. Tracy for preparation of the photomicrographs.

* The fine structure of the blood curve is being examined in present experiments. Although it is not yet possible to assign a precise value for the half time of segmented neutrophils in blood, present results based on daily sampling reveal a maximum of 12 hours with the suggestion of a half time of the order of 6 hours in dogs studied at more frequent intervals. These findings are germane to the question of an exchangeable peripheral pool which may be even smaller than described in the text.[13]

as revealed by labeling with C^{14} adenine.[11] A 3- to 4-day minimal time for development to the point of release from marrow is in good agreement with values for both dog and man based on P^{32} incorporation into DNA of leukocytes.[14-16] Comparable estimates of the time spent in marrow also have been made from *in vitro* observations of marrow suspensions[4] and from consideration of mitotic activity.[7]

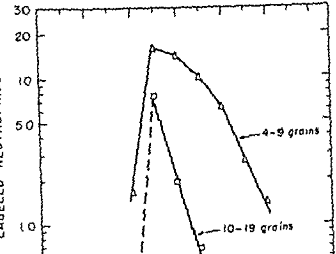


FIGURE 5. Disappearance of labeled neutrophils from blood. Each point is based on enumeration of 2000 neutrophils.

The disappearance of labeled neutrophils in peripheral blood, presented in FIGURE 5, is of particular interest. The data suggest that the half time for disappearance of all labeled cells from blood is about 2 days. In fact, however, a half time of 12 to 24 hours is apparent when consideration is given to more homogeneous classes of labeled cells as revealed by grain counts. Thus we may note the sharp difference in the initial decay of the 4 to 9 and 10 to 19 grain classes. We interpret this to mean that cells ... grains, which are derived mainly from progen... tinue to be released f... This

A METHOD FOR LABELING LEUKOCYTES WITH RADIOACTIVE DIISOPROPYL FLUOROPHOSPHATE (DFP[32])*

J. W. Athens,† A. M. Mauer,‡ Helen Ashenbrucker, G. E. Cartwright, M. M. Wintrobe

University of Utah College of Medicine, Salt Lake City, Utah

Introduction

For a better understanding of leukocyte pathophysiology it would be helpful if the leukocyte compartments and turnover rates could be measured in normal subjects, as well as in a variety of clinical situations. Numerous attempts to obtain this information have been made, but the studies have given contradictory results.

These results might be expected to differ for several reasons. First, different animal species have been used, and it is possible that leukocyte life span is not the same in all species. Also, a distinction must be made

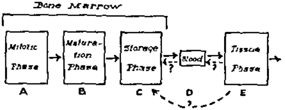


FIGURE 1. Schematic representation of the phases of leukocyte life span. The interrupted arrows represent possible but unproved leukocyte migrations. The interrupted arrow extending from the tissue phase directly to the storage phase is included to indicate the possibility that leukocytes may enter several tissues and be stored there as well as at the site of production.

between the life span of different cell types. For example, there is evidence that the life spans of granulocytes and lymphocytes differ.[1] Second, stress-producing procedures employed in the course of attempts to measure leukocyte life span such as anesthesia, surgery, or irradiation may have influenced leukocyte survival. Also, *in vitro* handling of leukocytes during labeling procedures may damage leukocytes.[2] Because of these considerations, certain reported data cannot be accepted, at least until they have been confirmed by more physiological measurements. Third, it has not always been appreciated that proliferation, maturation, storage, circulation, and periods of rest in tissues may represent phases of varying duration in the life span of a leukocyte (FIGURE 1). Attention must be given to the possibility that any

* The work reported in this paper was supported in part by Research Grants C 2231 and Cy 2339 from the National Cancer Institute and by a graduate training grant (2A 5098) from the National Institute of Arthritis and Metabolic Diseases, Public Health Service, Bethesda, Md.

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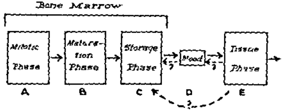


FIGURE 1 Schematic representation of the phases of leukocyte life span The interrupted arrows represent possible but unproved leukocyte migrations The interrupted arrow extending from the tissue phase directly to the storage phase is included to indicate the possibility that leukocytes may enter several tissues and be stored there as well as at the site of production

between the life span of different cell types For example, there is evidence that the life spans of granulocytes and lymphocytes differ [1] Second, stress-producing procedures employed in the course of attempts to measure leukocyte life span such as anesthesia, surgery, or irradiation may have influenced leukocyte survival Also, *in vitro* handling of leukocytes during labeling procedures may damage leukocytes.[2] Because of these considerations, certain reported data cannot be accepted, at least until they have been confirmed by more physiological measurements Third, it has not always been appreciated that proliferation, maturation, storage, circulation, and periods of rest in tissues may represent phases of varying duration in the life span of a leukocyte (FIGURE 1). Attention must be given to the possibility that any

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given experimental method measures only a fraction of the total leukocyte life span

From this it would seem that the most physiological approach yet devised to estimate leukocyte life span and production rate is the *in vivo* labeling of leukocytes with C^{14} or P^{32} compounds incorporated into cellular nucleic acids However, this technique has not been used widely because of the lengthy and complex procedures involved in the isolation of nucleic acids and the large amount of blood required Furthermore, because there is reutilization of the radioactive label, interpretation of results is difficult.

The purpose of this paper is to present a method for labeling leukocytes with radioactive diisopropyl fluorophosphate (DFP^{32}). This method has several advantages: (1) the technique is simple and accurate and requires the use of relatively small samples (20 ml) of blood; (2) the DFP^{32} binds rapidly and irreversibly to leukocytes, (3) the label is not reutilized,[3] and (4) the leukocytes are labeled *in vivo* and thus damage to leukocytes through handling *in vitro* is avoided

A more detailed account of this work will be presented elsewhere [4]

Method

Two mg of DFP^{32} (1 mg./ml in propylene glycol) are diluted with isotonic saline and injected intravenously over a 10-min interval Twenty-ml samples of venous blood are obtained at appropriate intervals thereafter and the leukocytes are isolated by a modification of the dextran sedimentation procedure,[5] followed by gramicidin-lysolecithin hemolysis[6] to remove remaining erythrocytes Thrombocytes are then removed by differential centrifugation The leukocytes so obtained are pipetted onto a scintillating plastic square The sample is dried and a second planchet is placed over the sample The leukocytes are thus sandwiched in the center of a plastic scintillation crystal The crystal is then placed on a standard phototube housed in a light-tight lead shield, and the amount of radioactivity is determined After counting is completed, the leukocyte sample is transferred to a flask for nitrogen determination by the micro-Kjeldahl method [7] A correction is made for physical decay and the leukocyte radioactivity is expressed as counts per min (cpm) per mg of white cell nitrogen.

Evaluation of the Method

Since DFP^{32} also labels erythrocytes and platelets, the leukocytes isolated must be free of these elements We have found that the procedure described above removes 99 9 per cent of the red cell radioactivity and over 93.5 per cent of the platelet radioactivity.

The efficiency of the counting system is about 75 per cent with a reproducibility within ±3 per cent With the amounts of leukocytes isolated, variation due to self-absorption is negligible and the over-all reproducibility of the method, as determined on duplicate samples, is within ±10 per cent No toxic effects attributable to DFP have been observed in about 60 human subjects given 2 mg of the compound intravenously

The evidence obtained to date indicates that elution of the label from

leukocytes does not occur to an appreciable degree when the cells are labeled *in vivo* However, additional studies will be necessary before it can be stated that no elution occurs The possibility has been considered that the DFP may injure the leukocytes and thereby cause a more rapid removal of the labeled cells from the circulation than occurs under normal circumstances. We have considerable evidence against this possibility Studies on the motility, phagocytic capacity, and oxygen consumption of leukocytes labeled with this compound have failed to reveal alterations from the normal

Radioactivity Curve Obtained in Normal Human Subjects

A representative radioactivity curve is presented in FIGURE 2 The curve can be divided into 3 phases an initial rapid decrease in radioactivity during

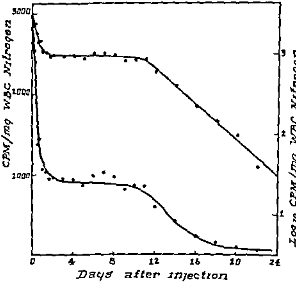


FIGURE 2 The mean leukocyte radioactivity curve of 7 normal subjects The data are shown as cpm/mg of WBC nitrogen and as the logarithm of cpm/mg of WBC nitrogen

data it is apparent that the results have been reasonably reproducible in normal subjects

Interpretation of the radioactivity curves in terms of leukokinetics is impossible at the present time The curves are complex, possibly due to

simultaneous labeling of the mitotic, storage, and circulating pools The possibility has been considered that DFP[32] labels several cell types, each of which turns over at an independent rate, the normal leukocyte radioactivity curve thus would represent a composite of several survival curves This, however, is not plausible since, in normal subjects, the only cell types present in sufficient numbers to affect the radioactivity curve in this way are the neutrophilic granulocytes and the lymphocytes. We have found that lymphocytes bind little or no DFP[32]. Therefore, it seems justifiable to conclude that the DFP[32] radioactivity curves represent the turnover of granulocytes in the circulation.

TABLE 1
SUMMARY OF THE DATA IN 25 NORMAL MALE SUBJECTS

Phase	Mean	±1 S D.	Range
I (T ½, hours)	6	2 2	3–11
II (Duration, days)	12	1 7	9–16
III (T ½, days)	3	0 52	2–4

Several possible [illegible] the [illegible] of [illegible] it [illegible] o elucidate the correct interpretation of these curves are now in progress in this laboratory

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BLOOD CELL SURVIVAL IN TISSUE CULTURES*

Edwin E Osgood

Division of Experimental Medicine, University of Oregon Medical School, Portland, Ore

A more appropriate title for this paper would probably be "Methods for the Determination of the Length of Life of Blood Cells in Culture," since the types of cells and the methods now available will be stressed rather than any survival values so far determined The blood cells include the erythrocytic and thrombocytic series and six major varieties of leukocytes These varieties are the granulocytic series, with its subdivisions into neutrophilic, eosinophilic, and basophilic, the lymphocytic series, the monocytic series, and the plasmocytic series

TABLE 1
AVAILABLE LONG TERM CULTURES OF HUMAN HEMIC CELLS

	Single source			Clones*	
Identification	J96	J111	J128	J111ct	J96c18
Date isolated	4 7 54	8/31/54	9/2/55	1957	1957
Source blood = B, marrow = M	B	B	B	J111	J96
Patient	OSK112	OSK222	OSK26		
Age, sex	26, M	25, F	59, F		
Blood type	A Rh+	A Rh−	O Rh+		
Diagnosis	ALML	ALML	CLGI		
Cell series					
Predominant	Mono	Mono	Gran	Gran (?)	Mono
Others	Gran Erythro	Gran Erythro	Mono Erythro		

* Isolated by the method of Theodore Puck, Biophysics Department, University of [illegible] to Leone S St Vincent of the [illegible] W Brown, Carver Foundation, [illegible]

TABLES 1 and 2 list the essential data on the cultured strains of human

simultaneous labeling of the mitotic, storage, and circulating pools The possibility has been considered that DFP[32] labels several cell types, each of which turns over at an independent rate; the normal leukocyte radioactivity curve thus would represent a composite of several survival curves This, however, is not plausible since, in normal subjects, the only cell types present in sufficient numbers to affect the radioactivity curve in this way are the neutrophilic granulocytes and the lymphocytes We have found that lymphocytes bind little or no DFP[32]. Therefore, it seems justifiable to conclude that the DFP[32] radioactivity curves represent the turnover of granulocytes in the circulation.

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I (T ½, hours)	6	2 2	3-11
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Several possible kinetic models can be constructed that would explain the granulocyte radioactivity curve. Unfortunately, at the present time all of these hypotheses are without experimental support; experimental data that could lead one to reject them are equally lacking. Studies designed to elucidate the correct interpretation of these curves are now in progress in this laboratory

References

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2 [illegible] 1951 The role [illegible]
3. [illegible] of [illegible]
4 [illegible] te [illegible] label In preparation
[illegible] and phyto- [illegible]
7 Kabat, E. A & M M [illegible] Experimental Immunochemistry : 282 Thomas Springfield, Ill

normal precursors. The granulocytic cells retain some alkaline phosphatase if stained by the method of Kaplow,[7] but have lost their peroxidase. All other cell types are phosphatase-negative.

In each of these series of hemic cells the length of life may vary with the population present at the time of study. Freshly isolated cells from a healthy adult will have a range of survival times, and these may differ also with age, with many environmental factors related to the culture technique and, of course, they will be different for each species. I have determined values for only a few of these numerous variables, since I have concentrated my efforts on making available for study each of the cell varieties that may be derived from normal or leukemic human blood.

To understand this paper it is necessary to have clearly in mind my concept of the methods of cell division that occur in man and other metazoa,

TABLE 3
DEFINITIONS AND RELATIONS

t = time in hours

α = number of cells capable of division, potentially immortal. Each may start a colony

α_0 = cells at time 0 = time culture placed in incubator or DNA label added

b, c, d = recognizable stages of cells with a finite life span which differentiate and die

z = dead cells present which are not yet completely autolyzed

T_z = time from cell death to complete autolysis

n = cells of categories b through z present at any one time

λ = total cells of categories b through z produced in time t from α_0

Z = all cells derived from α_0 that have disappeared since time t_0

s = total cells present at any one time = $n + \alpha$

S = total of all cells = $\alpha + \lambda$. Does not include n cells present at time t_0

M = duration of mitosis or its phases. M_p, M_m, M_a, and M_t

MI = mitotic index $\frac{\alpha_M}{s} = \frac{M}{g} \cdot \frac{s}{\alpha}$

I = intermitotic interval. Time from end of one mitosis to beginning of another in same cell

g = generation time including all types of divisions = $M + I$

g_α = generation time for α cells

g_n = generation time for n cells

T = life span of n cell from division to death

T' = $g + T + T_z$ = "life" span of n cell, which is measured by DNA labeling

λ = number of divisions per α cell = $\frac{S - \alpha_0}{\alpha_0} = \frac{S}{\alpha_0} - 1 = \frac{t}{g}$ for $t < g_\alpha$

λ_α = number of α cell producing divisions per α_0 cell since t_0 = $\frac{\alpha_0 2^{\frac{t}{g_\alpha}} - \alpha_0}{\alpha_0} = 2^{\frac{t}{g_\alpha}} - 1$

$\lambda_N = \frac{\lambda}{\alpha_0}$ at g_α = λ cell producing divisions per α cell in time $g_\alpha = \frac{g_\alpha}{g} - 1$

$\lambda_n = \frac{g_\alpha}{T' - g} = \frac{1}{\alpha_0} \frac{\lambda}{\Sigma_0^t \alpha} = \frac{\lambda_N(T' - g)}{g_\alpha} = \frac{\lambda_N(T + T_z)}{g_\alpha}$ = mean number of n cell producing divisions per α cell during mean "life" span of n cell

Each of these variables will have a probability distribution but also will have a mean value.

TABLE 2
AVAILABLE LONG-TERM CULTURES OF HUMAN HEMIC CELLS

	Mixed cultures*					
Identification	M166 J111	J169 J129	J173, J111	J181, J111	J184, Ca†	J189, J111a
Date isolated	2/12/57	3/15/57	5/29/57	10/21/57	12/6/57	12/18/57
Source blood = B marrow = M	B	B	B	B	M	B
Patient	OSK1063	MSH244304	MCH241428	OSK1228	MCH84010	OSK1198
Age sex	65 M	1, F	9, F	79, M	51, M	64 M
Blood type	B Rh+	O+	O Rh+	A Rh+	O Rh+	O Rh+
Diagnosis	CLLL	ALLL	CLGL	ASPL	Erythro-leukemia	ALBL
Cell Series						
Predominant	Lymph	Lymph	Erythro	Plasmo	Erythro	Baso
Others			Gran		Ca† Fibrocytic	

* Only new hemic source is described, for description of culture listed second see TABLE 1

† This culture contains cells resembling a cuboidal epithelial malignant tumor seen in the original marrow, but no primary could be found at necropsy

Seed cultures from these strains are available from R W. Brown (see TABLE 1)

at infrequent intervals only when there is a high population density and to use large [illegible] method[2]

the subscript indicates the days in culture since original isolation at the start of an experiment. The second culture number, for the mixed cultures, indicates the long-term culture[2] to which the cells were added

I have discovered recently that, for cells requiring a very high gradient factor, the addition of the new cells to a rapidly growing well-established culture greatly facilitates initial isolation This possibly explains why Bichel[3] and deBruyn[4] were able to grow the lymphocytic series from rodents successfully where others had failed, and possibly also the principle underlying the feeder-layer technique, of Puck *et al* [5] for isolating clones The proportions of the different types of cells may be varied at will by varying the gradient factor [1] The cells will grow in almost any of the fluid media [illegible] using NCTC-109 or Eagle's medium, [illegible] nd human serum, 10 to 20 per cent, [illegible] balanced salt solution equilibrated [illegible] the pH at 7 0 ± 0 2 I subculture when there are about 4 to 5 × 10³ cells/sq mm of surface by adding 1 ml of aqueous 1 5 per cent disodium ethylene diamine tetracetate (E D T.A)/ [illegible] and [illegible] illu-

As might be expected from my unifying concept of cancer,[6] the life spans of these cells become progressively shorter the longer they are cultured, and only the more immature of the differentiating cells remain in these cultures. All acquire malignant characteristics even if they are derived from

finite life span The life span of the alpha cell may be defined either as infinite or as one doubling time (g_a) for a large population over a long period of time It is the existence of this alpha cell that makes tissue culture,

TABLE 5
MEANS OF DETERMINING ESSENTIAL VALUES

s = total cell count
[illegible]
[illegible]
[illegible]
[illegible]
[illegible]
T_s = time initial rate of cell decrease intercepts α_0
$n = S - \alpha$

marrow transplant, and the maintenance of transplantable tumors possible In fact, however, there is some mortality of alpha cells due to lethal alterations in the genetic material of the cell and other variables

Methods Available for Determining the Length of Life of Cells in Culture

These methods are listed in TABLE 6. Many of them are applicable *in vivo* as well as to tissue culture They have been reviewed recently by Lissac[11] and by Osgood[10] with some discussion of their advantages and limitations, only specific applications to culture will be discussed here

TABLE 6
METHODS FOR DETERMINING LEUKOCYTE LIFE SPAN IN CULTURE

(1) Rate of decrease with no division
(2) Relative proportion of stages and disintegrated cells
(3) Rate of incorporation of label in DNA
(4) Counting cells in cloned colonies
(5) Counting chains and cells in chains
(6) Mitotic index with or without mitostatic agents
(7) Time lapse phase photomicrograph of colony formation
(8) Chemostat

Method 1, the first employed,[12, 14] gave a length of life for the n neutrophils of 24 to 96 hours, mean 48, for the n eosinophils of about 8 days, and for the n basophils of about 12 days It consists essentially in starting a series of cultures from fresh normal blood and following the time course of disappearances of the n cells which are incapable of division I would recommend using the inverted slide cap[7, 15] with a cover slip instead of a slide for this procedure A cover slip has less tendency to slip while being affixed than does a slide, and it permits better observation with an upside down microscope of the cultures during life Using this technique, it has been possible to demonstrate that at least some of the lymphocytes of normal blood,[10] as well as the promonocytes and proplasmocytes, retain their ability to

described in previous papers [4, 8-10] The figures and graphs in this paper should further clarify this concept. The symbols and definitions used in this paper are given in TABLE 3 and are shown graphically in FIGURE 1*

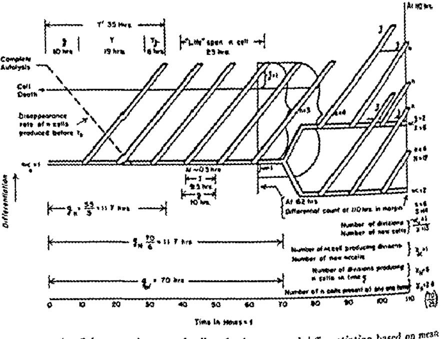


FIGURE 1 Schematic diagram of cell multiplication and differentiation based on mean values for monocytic series from the J111 cells in culture M166, J111 Time data were derived from tritium-labeled thymidine experiment 1 See FIGURE 3 and text

TABLE 4
EQUATIONS

$$\alpha = \alpha_0 \ (X_\alpha + 1)$$
$$N = \alpha_0 \ X_N \quad X_n = X_N(\alpha - 1)$$
$$S = \alpha_0 \ X_N \ (X_\alpha + 1) = X_N \ \alpha(\alpha - 1) = \alpha + N - Z = \alpha + n = \alpha_0(X + 1)$$
$$Z = N \text{ at } (t - g_\alpha) + T'$$
$$n = N - Z = \alpha X_n$$
$$s = S - Z = \alpha + n = S \text{ for } t = T'$$
$$z = n \ \frac{T_z}{T'}$$

The equations are given in TABLE 4, and methods of determining each of the essential values are presented in TABLE 5 The potentially immortal cells that have been called *a* cells in previous papers are here called alpha cells for increased clarity and to separate them further from the *n* cells, which have a

* This figure is presented before the basis for the time values in it are presented because it aids in understanding the symbols, definitions, and equations Cylinders are used for single cells to show the continuity in time and stage of differentiation of the cell

calibrated so that the final count can be determined in cells per sq mm, counting enough complete Miller disk fields to have 50 to 100 cells from each of the 13 areas, and averaging the computed count for 1 sq mm from each area. This procedure corrects for any unequal distribution that may have been due to convection currents A line through an arithmetic plot of the points at the intercepts of time and number will give the value for T' at the zero intercept for the n cells or their stages as found in the circulating blood under the environmental conditions in culture and probably will give the

probably give the best estimate of the time spent in each stage of differentiation and as a dead cell The times T_b, T_r, , T_z are proportional to the numbers of cells n, as may be seen by drawing horizontal lines through FIGURE 1 to indicate the proportions of cells at each stage of differentiation These various times will be proportional to the numbers just as $\frac{z}{n} = \frac{T_z}{T'}$ This has been determined only for the neutrophils,[14, 15] and it gives values of the order of 12 hours for the myelocytes, 12 hours for the metamyelocytes 36 hours for band cells, 48 hours for segmented cells, 108 hours for total intact neutrophils, and 18 hours for disintegrated neutrophils The life span of the segmented neutrophil of chronic granulocytic leukemia was shorter This accords with the concept that the essential change in malignancy is a shortening of the life span of the n cell [6]

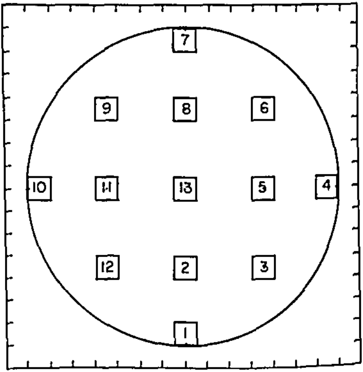
Method 3 in TABLE 6 is the method that has been most used It consists in determining the rate of incorporation of a specific label for DNA The equations for such incorporation required by my concept of cell division, however, are far different from those ordinarily used These equations have been worked out in detail by my associate, Rigas [17] Cultures have the advantages in DNA labeling that there is no absorption or excretion, the number of possible pools is greatly reduced, and it is somewhat easier to determine the varieties of cells included in the material analyzed Cloned cultures may be used, and the colony pattern[18] and histochemical stains aid in an accurate differential count Cells receive little trauma in falling on a cover slip through a viscous fluid, therefore disintegrated cells may be reliably interpreted as cells that have died recently

in collaboration with Victor P Bond and E P Cronkite of the Brookhaven National Laboratory, Upton, N Y John Brooke, the cytologist working with me, added 0 5 μc per ml of tritium-labeled thymidine to a mixed $M100_{62}J111_{165}$ culture with a cell count of 100 per cu mm, and introduced 3 5 ml of this cell suspension into each of many replicate inverted cover slip cultures, giving a cell count of 2000 over each sq mm of surface, since 3 5 ml forms a layer 20 mm deep in the Kahn shell vials we use Four

SCALE: 1 cm = 1mm

Diameter of vial = 14 to 15 mm.

Area = 150 to 178 sq mm.



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5 dead,

calibrated so that the final count can be determined in cells per sq mm, counting enough complete Miller disk fields to have 50 to 100 cells from each of the 13 areas, and averaging the computed count for 1 sq mm from each area. This procedure corrects for any unequal distribution that may have been due to convection currents. A line through an arithmetic plot of the points at the intercepts of time and number will give the value for T' at the zero intercept for the n cells *or their stages as found in the circulating blood* under the environmental conditions in culture and probably will give the *most reliable approach possible by culture methods to that occurring in life* for the particular individual of the particular species investigated.

Method 2,[13, 14] in TABLE 6, which may be combined with Method 1, will probably give the best estimate of the time spent in each stage of differentiation and as a dead cell. The times T_b, T_c, . . . , T_z are proportional to the numbers of cells n, as may be seen by drawing horizontal lines through FIGURE 1 to indicate the proportions of cells at each stage of differentiation. These various times will be proportional to the numbers just as $\frac{z}{n} = \frac{T_z}{T'}$.

This has been determined only for the neutrophils,[13, 14] and it gives values of the order of 12 hours for the myelocytes, 12 hours for the metamyelocytes, 36 hours for band cells, 48 hours for segmented cells, 108 hours for total intact neutrophils, and 18 hours for disintegrated neutrophils. The life span of the segmented neutrophil of chronic granulocytic leukemia was shorter. This accords with the concept that the essential change in malignancy is a shortening of the life span of the n cell.[6]

Method 3 in TABLE 6 is the method that has been most used. It consists in determining the rate of incorporation of a specific label for DNA. The equations for such incorporation required by my concept of cell division, however, are far different from those ordinarily used. These equations have been worked out in detail by my associate, Rigas.[17] Cultures have the advantages in DNA labeling that there is no absorption or excretion, the number of possible pools is greatly reduced, and it is somewhat easier to determine the varieties of cells included in the material analyzed. Cloned cultures may be used, and the colony pattern[18] and histochemical stains aid in an accurate differential count. Cells receive little trauma in falling on a cover slip through a viscous fluid, therefore disintegrated cells may be reliably interpreted as cells that have died recently.

Of the many labels available, tritium-labeled thymidine has the advantage that radioautographs can be made at each time interval from replicate cover slips and the absolute number and the number labeled of each cell type per sq mm determined as shown in FIGURE 3, which is from an experiment done in collaboration with Victor P. Bond and E. P. Cronkite of the Brookhaven National Laboratory, Upton, N. Y. John Brooke, the cytologist working with me, added 0.5 μc per ml of tritium-labeled thymidine to a mixed $M106_{(62)}J111_{(63)}$ culture with a cell count of 100 per cu mm and introduced 3.5 ml of this cell suspension into each of many replicate inverted cover slip cultures, giving a cell count of 2000 over each sq mm of surface, since 3.5 ml forms a layer 20 mm deep in the Kahn shell vials we use. Four

or more of these cultures were sampled at 4, 12, 24, 48, 72, and 120 hours, with the results diagramed in FIGURE 3. Fixed but unstained preparations were mailed to Bond and Cronkite, who made radioautographs, using the stripping film technique[19] with a Feulgen stain, and they mailed replicates back to us. The absolute numbers of cells of each type and the numbers labeled were computed as the average per sq. mm. to determine the points from which FIGURE 3 is constructed, using the same areas for the counts as diagramed in FIGURE 2. Each point in the figure for the monocytic series

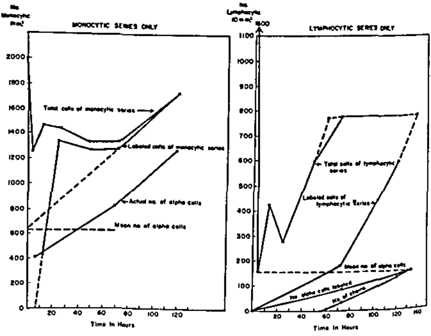


FIGURE 3 Graphic analysis of tritium-labeled thymidine experiment 1, based on data from culture $J111_{1059}M166_{162}$ See text for explanation

is the average of 900 or more cells counted on each of 2 or 3 replicates so that the 95 per cent confidence limits for each point should be less than 10 per cent of the value.

Since each of the points for the lymphocytic series was based on counting between 300 and 400 cells, the 95 per cent confidence limits should be less [illegible]

any of the cultures in which one cell was labeled and the others were unlabeled There were a few chains of 2 cells labeled in the 48-hour culture; many chains of 2 to 4 were labeled in the 120-hour culture, as illustrated in FIGURE 4

The equations from which the values for the monocytic series and lymphocytic series were derived are given in TABLE 4 and are shown as graphic solutions in FIGURE 3

FIGURE 1 illustrates what would happen if all alpha cells behaved as the mean of this culture did, and TABLE 7 shows numerically what would happen through 3 generation times. Note that with large populations, since the

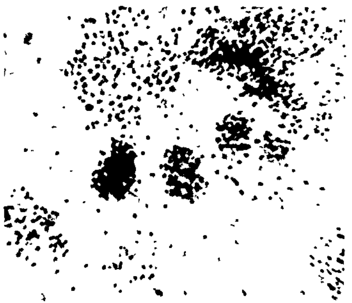


FIGURE 4 Photomicrograph from tritium-labeled thymidine experiment 1 [illegible]

cell next to it, the second n cell produced, and the cell next to the alpha cell as the last n cell produced by division of the alpha cell This would account for the progressive decrease in intensity of labeling from left to right The intense labeling in the upper right is another chain of lymphocytes in which the nuclei are in poor focus and are superimposed on the nucleus of a cell of the monocytic series

value g_a may be so staggered that one alpha cell will wait a full g_a before becoming 2 alpha cells, others may divide into 2 alpha cells almost immediately, and still others at any point between, so that the mean values for large populations given in TABLE 7 approximate what happened in this culture From TABLE 7 it may be seen that the value of s may fluctuate through a range of about 1 5 to 4 times the number of alpha cells present and that with a longer relative value of T' the upper limit of the ratio, $\frac{s}{\alpha}$, could be

or more of these cultures were sampled at 4, 12, 24, 48, 72, and 120 hours, with the results diagramed in FIGURE 3. Fixed but unstained preparations were mailed to Bond and Cronkite, who made radioautographs, using the stripping film technique[19] with a Feulgen stain, and they mailed replicates back to us. The absolute numbers of cells of each type and the numbers labeled were computed as the average per sq. mm. to determine the points from which FIGURE 3 is constructed, using the same areas for the counts as diagramed in FIGURE 2 Each point in the figure for the monocytic series

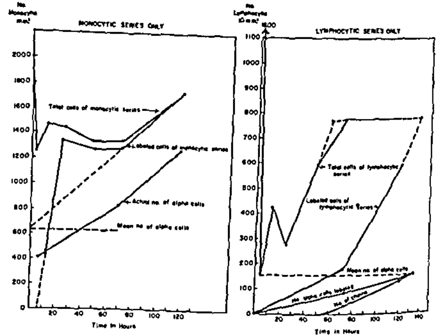
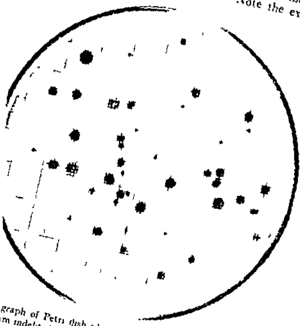


FIGURE 3 Graphic analysis of tritium-labeled thymidine experiment 1, based on data from culture $J111_{1058}M166_{142}$ See text for explanation

is the average of 900 or more cells counted on each of 2 or 3 replicates so that the 95 per cent confidence limits for each point should be less than 10 per cent of the value.

Since each of the points for the lymphocytic series was based on counting between 300 and 400 cells, the 95 per cent confidence limits should be less than 25 per cent of the value given, which is that for 10 sq mm, or 10 times the scale for the monocytic series Donald Jenkins of our laboratory did most of these counts. Few tritium-labeled cells were observed in the lymphocytic series before 48 hours No chains of lymphocytes were observed in

ll can produce a colony FIGURE 5 is a photograph of a Petri dish made
m Clone 1 of $J111_{1156}$ in Puck's laboratory This Petri dish was seeded
October 29, 1957, and fixed and stained on November 11, 1957 from
ne 1 of J111 This clone is interpreted as having been derived from
nally normal cells of the granulocytic series because the cells are phos-
ase-positive by the method of Kaplow [7] Note the extreme range in



graph of Petri dish plated from culture of $J111_{1156}$ after 14 days'
im indebted to Theodore Puck of the Biophysics Department of the
lo Medical Center, Denver Colo Note the extreme variation in
onies See FIGURE 6 and the text for total distribution of colonies
ow of the rim of the Petri dish

lonies Because of this variation in colony size, I exam-
g microscope 13 areas that to the naked eye appeared to
and I discovered 19 colonies in these areas From the
determined from the field size of the dissecting micro-
at 121 colonies were to be expected on the total area
placed the plate on mm paper as shown in FIGURE 5
microscope, counted the entire area of the plate, deter-
ells in each colony There were 14 colonies of 1 cell,
tal of 60 colonies invisible to the naked eye. The
d 118 cells There were 60 colonies ranging in size
est visible colony, to 39,460 cells, computed as the

quite high For example, I calculated it as 184 for the erythrocytic series in the steady state in adult man.[4] If T' is shorter than g, single cells may be observed that are labeled but still have formed no colonies, but continue to produce n cells which die and are completely autolyzed

TABLE 7
DEFINED VALUES USING NUMERICAL DATA FROM MONOCYTIC SERIES CULTURE
J111[illegible]M166[illegible]

For individual colonies														For large populations	
t hours	$\frac{t}{g}$	$\frac{t}{g_\alpha}$	α	N	S	Z	s	$\frac{t}{g_\alpha}$	α	N	S	Z	s	s	$\frac{s}{\alpha_0}$
0	0	0	$1 = \alpha_0$	0	1	0	1	0	1	0	1	0	1	2	1
10 = g	1		1	1	2	0	2	1	2	1	3	0	3	5	2.5
20	2		1	2	3	0	3		2	3	5	0	5	8	4.0
30	3		1	3	4	1	3		2	5	7	1	6	9	4.5
40	4		1	4	5	2	3		2	7	9	3	6	9	4.5
50	5		1	5	6	3	3		2	9	11	5	6	9	4.5
60	6		1	6	7	4	3		2	11	13	7	6	9	4.5
70 = g_α	7	1	2	6	8	5	3		2	13	15	9	6	9	4.5
80	8		2	8	10	6	4	2	4	13	17	11	6	10	5.0
90	9		2	10	12	6	4		4	17	21	13	6	10	5.0
100	10		2	12	14	8	6		4	21	25	13	8	14	7.0
110	11		2	14	16	10	6		4	25	29	17	12	18	9.0
120	12		2	16	18	12	6		4	29	33	21	12	18	9.0
130	13		2	18	20	14	6		4	33	37	25	12	18	9.0
140	14	2	4	18	22	16	6		4	37	41	29	12	18	9.0
150	15		4	22	26	18	8	3	8	37	45	33	12	20	10.0
160	16		4	26	30	20	10		8	45	53	37	12	22	11.0
170	17		4	30	34	22	12		8	53	61	37	12	24	12.0
180	18		4	34	38	26	12		8	61	69	45	24	36	18.0
190	19		4	38	42	30	12		8	69	77	53	24	36	18.0
200	20		4	42	46	34	12		8	77	85	61	24	36	18.0
210	21	3	8	42	50	38	12		8	85	93	69	24	36	18.0
220	22		8	50	58	42	12	4	16	85	101	77	24	36	18.0

It would be interesting to see results, determined with one of the digital computers, of random walks within set limits and probability functions for the variables defined in TABLE 3

The rate of P^{32} incorporation in gradient bulk cultures,[2] when analyzed by the equations of Rigas,[17] will give mean values for DNA turnover rate under optimal conditions of growth Of course, many other labels, such as C^{14}-labeled thymidine or adenine or stable C^{13} or N^{15} labels as used by London *et al.*[20] could also be employed.

Method 4, in TABLE 6, either carried out by the technique of Puck *et al*[21] or adapted to the inverted cover slip vial procedure,[2] appears to be the best *method* for determining the number of alpha cells present. Only an alpha

cell can produce a colony. FIGURE 5 is a photograph of a Petri dish made from Clone 1 of J111₁₃₃₈ in Puck's laboratory. This Petri dish was seeded on October 29, 1957, and fixed and stained on November 11, 1957 from Clone 1 of J111. This clone is interpreted as having been derived from originally normal cells of the granulocytic series because the cells are phosphatase-positive by the method of Kaplow.[7] Note the extreme range in

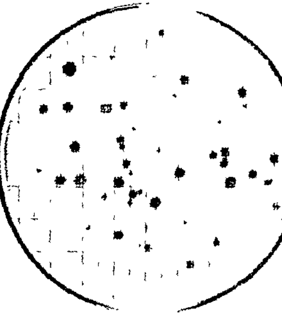
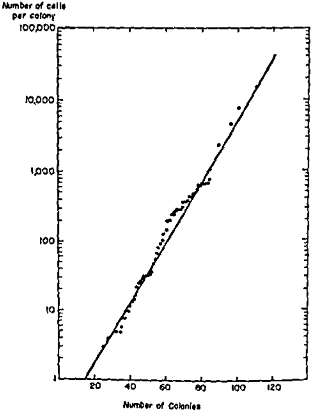


FIGURE 5. Photograph of Petri dish plated from culture of J111₁₃₃₈ after 14 days growth for which I am indebted to Theodore Puck of the Biophysics Department of the University of Colorado Medical Center, Denver, Colo. Note the extreme variation in size of the visible colonies. See FIGURE 6 and the text for total distribution of colonies. The oval line is a shadow of the rim of the Petri dish.

size of the visible colonies. Because of this variation in colony size, I examined with a dissecting microscope 13 areas that to the naked eye appeared to be free of colonies, and I discovered 19 colonies in these areas. From the total area examined, determined from the field size of the dissecting microscope, I computed that 121 colonies were to be expected on the total area of the plate. I then placed the plate on mm. paper as shown in FIGURE 5 and, using a dissecting microscope, counted the entire area of the plate, determining the number of cells in each colony. There were 14 colonies of 1 cell, 11 of 2 cells, and a total of 60 colonies invisible to the naked eye. The largest of these contained 118 cells. There were 60 colonies ranging in size from 134 cells, the smallest visible colony, to 39,460 cells, computed as the



[illegible] from culture [illegible]

largest. The actual numbers of cells in each colony were counted up to 804. For the larger colonies, the number of cells present in the colony was computed by counting the nuclei touching a line of one side of the Miller disk square across the greatest diameter of the colony. Using this number as the diameter of a circle in cell units, the area of that circle was taken as the cell count. This method was checked by actual counts on some of the

colonies between 100 and 800 cells, and gave very good agreement. In FIGURE 6 the distribution of the total 120 colonies is shown arranged in sequential order of size and plotted on semilogarithmic paper. Individual colonies are plotted up to 804 cells, and the number between powers of 2 from 1024 or 2^{10} to 16,400 or 2^{14}. There were 10 colonies found with more than 16,400 cells in each. Note that the slope of the line could have been predicted from the colonies with counts under 100 where there is essentially no error in counting. This remarkable distribution of colony size from 2^{0} to 2^{15} probably shows what really happens in these cultures and that the mean value is far from giving a complete picture. Using the equation given in FIGURE 6, I computed the expected size of each colony and, from these values computed that the largest 18 colonies contained 90 per cent of the cells, that the smallest 74 colonies contained less than 1 per cent of the cells, and that the total number of cells, 413,500, on the plate divided by 120 gave a mean of 3450 cells per colony, which is $2^{11.76}$. This would give a value for g_a of 1.19 days, or 28.6 hours. The range of g_a values was from 22.1 hours for the largest colony to more than 14 days for the single cells.

My interpretation of the smaller colonies was initially the same that was offered by Puck, namely that a few cells had been displaced from the largest colonies. However, the demonstration that the small and large colonies belonged to the same random distribution and the good correlation between the number of colonies observed between each power of 2 and that predicted from the equation make it almost certain that each colony started from a single cell distributed at random on the plate. Furthermore, the equation could have been derived from the distribution of either the small or large colonies, the small colonies were similar in morphology to the larger colonies, the one- or two-cell colonies looked just as healthy as the larger colonies. My interpretation of the single-cell colonies is that they have been producing n cells with a shorter life span T' than g and happen to have been fixed at a time when no unautolyzed n cells were present.

Since these cells had been selected by our subculturing technique[3] for rapid growth ever since their original isolation and since they were derived from a relatively recently cloned subculture, to me this means that evolution is going on in these cultures and colonies. Very quickly there is again a wide distribution in the value of g_a, in the number of n cells produced, and in their life span, T'.

This experiment suggested that I might be able to combine the technique of Puck *et al.*[21] with the tritiated thymidine technique[19] in my inverted cover slip cultures[3] by setting up many replicates with an inoculum of 100 to 400 cells in the total 3.5 ml. of culture in the vial and examining some at frequent intervals. Preliminary work with this technique suggests that it offers great promise of being extraordinarily useful. See FIGURE 7 and TABLE 7 for results of our first study with culture M160$_{165}$J111$_{1767}$. The entire area on which the cells could settle is counted with as much magnification as needed, using the mechanical stage and the Miller disk. With this technique there is essentially no error either in determining the number of colonies or the number of cells in each colony. The thymidine labeling

serves as an absolute control on whether the cell is alive at the start of the experiment and as to whether a dead cell resulted from a division after the tritium-labeled thymidine was added. Both the mean value and the individual value for each colony for each of the variables defined in TABLE 3 can be computed. Enough replicates can be used for each time interval to reduce the statistical error to any desired confidence limits.

This combined technique should be equally applicable to the study of any cell series that could be grown in suspension cultures and would be particularly suitable for a study of the various ascites tumors. It also could be used

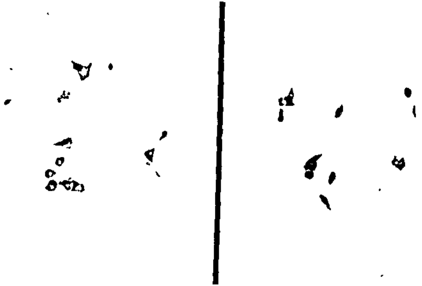


FIGURE 7. Two monocytic colonies from tritium-labeled thymidine experiment 2, 192-hour sample, as seen in Giemsa stains. Note remnants of dead cells and ease with which number of colonies and number of cells in colony could be counted.

for studying freshly isolated cells from various tissues if conditions can be [illegible]
ils
to
form any colonies, although living single cells of the lymphocytic series were always present.

Note from TABLE 7 the considerable range in colony size for the monocytic series. The number of cells in individual colonies ranged from 1 to 21 with many intermediate odd, as well as even, numbers between powers of 2 [illegible]
e living
the cor-
d,[19] but
e funda-

It is interesting to note that in some of these cultures a few isolated nucleated and nonnucleated erythrocytes with adequate hemoglobin present were observed, but again no colony formation was noted, suggesting the great importance of a high gradient factor for growth of these cells [15]

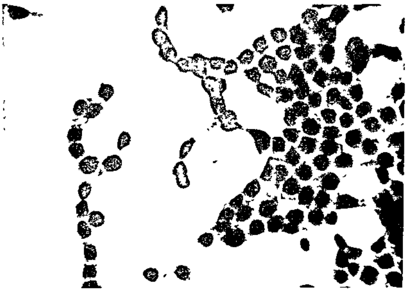


FIGURE 8 Lymphocytic colony from culture $M166_{45}J111_{945}$ to show chain formation

autolysis in a matter of a few hours and gives an estimate of n for the initial inoculum

Method 5, shown in TABLE 6, has not been previously described The character of the tritiated thymidine labeling of the cells from chronic lymphocytic leukemia, FIGURE 4, suggests that for the lymphocytic and erythrocytic series of cells,[1] which tend to grow in chains, it may be possible to compute the number of alpha cells from the cells at one end and at the branches of a chain, and the number of n cells from the number of cells in

Method 6, in TABLE 6, the mitotic index on acetocarmine-stained cover slips from inverted vials with or without addition of mitostatic agents, may be used alone or combined with the previous methods. The mitotic index has been much used in other species and *in vivo*.[22, 23] FIGURE 9 illustrates a photomicrograph from such an acetocarmine stain on a cover slip from an inverted vial culture of M166[illegible]J111[illegible]. This preparation was made 144 hours after 3.5 ml [illegible]
in the cover-slip-ca[illegible]
of 446 cells per sq[illegible]



FIGURE 9. Acetocarmine stain of culture M166[illegible]J111[illegible] at 144 hours to show the suitability of this type of preparation for mitotic index determination. All cells in this field are typical of the monocytic series. Cells of the lymphocytic series are much smaller.

giving a mitotic index of 0.04, which would mean a doubling time of 25 hours, if all the cells were alpha cells and mitosis lasted about an hour, rather than the 70 hours observed for the doubling time or the 10 hours derived from tritiated-thymidine labeling. But if 28 alpha cells per 100 are correct, as derived from TABLE 8, then one seventh of the alpha cells are in process of mitosis at any one time, and reasonable agreement is obtained with the [illegible]

TABLE 8
M166₁₆₅J111₁₆₄ TRITIUM LABELED THYMIDINE EXPERIMENT NO 2

Hours	s Total cells	s − 2 Living cells	z Dead cells	Single cells	Colonies	α₀ Potential colonies	No cells in colonies 2	3	4	5	Larger
0	100	82	18								
3	51	21	30	16	2	18	1	1			
4	79	29	50	21	6	27	6				
8	36	18	18	14	2	16	2				
12	41	19	22	19	0	19	0				
16	81	32	49	30	1	31	1				
24	69	31	38	27	2	29	2				
48	66	35	31	25	5	30	5				
72	148	45	103	26	7	33	4	1	1	1	
96	131	32	99	20	6	26	6				
120	118	40	78	21	10	31	8	2			
192	140	114	26	10	19	29	5	5	3	2	8, 11, 17, 21
264	107	90	17	11	18	30	9	2	2	1	6, 7, 12, 17
384	138	64	74	18	16	34	8	2	1	1	6, 6, 8, 57
385	111	66	45	19	16	35	9	1	2	1	8, 13, 16

Mean	28 ± 5
Lymphocytic	2
Monocytic	26

Method 7, in TABLE 6, time-lapse-phase cinephotomicrography of single cells or small colonies in a Rose chamber,[24] or double cover slips sealed on each side of a slide with a small hole in it, should make it possible to determine all of the values with extreme accuracy, providing ideal conditions for growth can be determined for such preparations Unfortunately, it is difficult to get a satisfactory gradient factor for growth and still have sufficiently few cells to give individual colony formation

Method 8, in TABLE 6, the use of a chemostatlike culture technique,[25, 26] should make it possible to determine rates of cell division under constant conditions It is tempting to speculate that food for the hungry could be provided by similar cell strains isolated from common farm animals grown in large-scale cultures similar to those used in the production of antibiotics if a sufficiently cheap source of nutrients could be found This should produce good animal protein with no bone, no fat, and no gristle and increase by a factor of 2^{20} or 1,000,000 times in a month or two

Variables Affecting Cell Survival in Culture

These are summarized in TABLE 9 Unfortunately they make it very difficult to interpret any of the observations except those by Methods 1 and 2 as having any real bearing on what is the actual state of affairs in the living individual of the species under study

TABLE 9
VARIABLES AFFECTING CELL SURVIVAL IN CULTURE

(1) Somatic genetic change.
(2) Gradient factor and population density.
(3) Composition of medium and frequency of change
(4) Physical factors temperature, surface area, and character of surface
(5) Ecology of the cell.

Variable 1, somatic, genetic change, is unquestionably occurring at a rapid rate in these cultures. In other words, evolution continues. These cells show wide variations in ploidy and are nearly all aneuploid. The doubling time, g_o, for culture J111 was about 60 days when first isolated versus 70 hours, or less, now more than 3 years later. The life span of each n cell has continued to shorten and the colony count experiments described show that these changes may occur very rapidly, as might be expected when one considers that in all of postnatal life in man there are only 5 doublings on the average,[9] whereas in tissue culture or in transplanted tumors there may be this number of doublings in a week or two, and also show that each mitosis offers some risk of genetic change. This also makes it improbable that it will ever be possible to prevent normal cells from becoming malignant in such cultures.

Variable 2, changes in gradient factor and population density, permits us to change at will the relative proportions of different cell types in the mixed cultures and the gradient factor changes with time in any culture technique except the chemostat methods.

Variable 3, while apparently less important than variable 2 within the range of composition of media currently in use, still has an almost infinite series of possible permutations, not to mention mutant nutritional requirements and metabolic waste product accumulation.

Variable 4, the physical factors of temperature, surface area, character of surface, pH, oxidation-reduction potential, CO_2 tension, and other factors that are regulated by the gradient factor and the size, shape, and location of the culture container and consistency of the medium could be studied in an almost infinite number of permutations.

Finally, the variables related to the ecology of the cell[15] are important. Certain cells appear to grow best in the company of other cells, and the malignant cell has a tendency to outgrow the corresponding normal cell. Even such a slight variation as whether fresh serum, which contains the unstable inhibitors,[8] or aged serum from the same source is used will make a difference. Saetren[27] and Rose[28] have shown that the inhibitors present seem to be specific for each fundamental cell type. Weatherley-Mein and Cottom[29] have shown that fresh transfusions depress the leukocyte count, while bank blood does not. These observations and the experiments herein recorded lend further support to my concept of the fundamental nature of growth[9] and malignancy[8] that were first presented[30] before The New York Academy of Sciences in May of 1954.

Since it is difficult to present clearly and concisely the results of 24 years

of study of the fundamental nature of growth and malignancy in relation to cell life span in one article, many will find it helpful to study at least references 1, 2, 6, 8, 9, and 10 as an aid in understanding this paper

Summary

Long-term cultures containing all varieties of human hemic cells except the thrombocytic series are now available from a central source for distribu-

a cloned population

Methods for study of cell growth and life span in cultures are outlined and some data on the life span of human hemic cells in culture are reported

The combination of radioautographs by tritium labeled thymidine, the Puck technique of colony counting, and my inverted cover slip-capped-vial culture method seems to offer great promise for quantitative studies of all

The methods of cell growth and division that I have suggested as being mathematically necessitated for multicellular organisms would seem to be the only mechanism that could explain the observations presented, and these observations lend further support to the unifying concept that the fundamental etiology of malignancy is an alteration of the genetic material of the alpha cell leading to early death of the *n* cell before it reaches the stage at which it normally produces a specific "feed back" inhibitor of logarithmic multiplication

The necessity for considering many variables, including the ecology of the cell *in vitro* or *in vivo*, in any study of cell growth and longevity is emphasized

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LEUKOCYTIC RESISTANCES IN PATIENTS FOLLOWING SPLENECTOMY AND THE RELATION BETWEEN THE SPLEEN AND THE VIABILITY OF WHITE BLOOD CELLS

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In this paper we describe the behavior of the osmotic and mechanical resistances of white blood cells in splenectomized patients for a variable period extending from a few months to nine years Splenectomy had been performed for diverse reasons in each individual case for hyperhemolysis, thrombopenia, portal hypertension, or traumatic rupture of the spleen A description of the techniques we used in determining the leukocytic resistances is to be found in our previous papers [1,2]

In these studies we have been able to note that in the 40 splenectomized subjects we examined the values of the leukocytic resistances (osmotic and mechanical) were decidedly superior to the normal average values, even in those cases in which the values of resistance prior to splenectomy were greatly inferior to the norm Such was the case also in those patients who had undergone removal of the spleen 4 to 8 years before for traumatic rupture of the spleen, that is, in subjects who therefore had had a normal spleen and in whom no hematopoietic defect played a role

In FIGURES 1 and 2 we compare the average curves of osmotic and mechanical resistances relative to splenectomized subjects with those pertaining to normal subjects The difference is evident, especially for those curves pertaining to the granulocytes

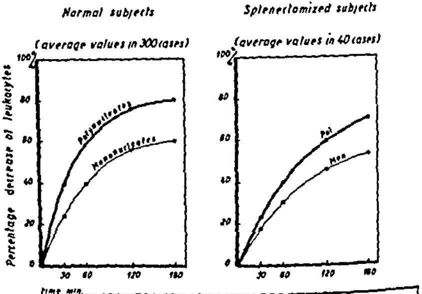
In FIGURE 1 the curves of resistance are obtained by plotting on the abscissa the time in which the leukocyte count is carried out and, on the ordinate, the percentage of white blood cell decrease at diverse time intervals after the initial count The resistance curves of polynucleates and mononucleates (lymphocytes and monocytes) are plotted separately On the left are plotted the curves of the average values of osmotic resistance of

resistance and the values of resistance of splenectomized subjects is statistically significant both for the granulocytes and mononucleates

In FIGURE 2 the curves of resistance are obtained by plotting on the abscissa the time in which the leukocyte count is carried out and, on the ordinate, the percentage of white blood cell decrease at diverse time intervals after the initial count The resistance curves of polynucleates and mononucleates (lymphocytes and monocytes) are plotted separately On the left are plotted the curves of the average values of mechanical resistance of granulocytes and mononucleates relative to normal subjects On the right the curves of the average values relative to the 40 splenectomized subjects are

plotted The difference existing between the normal average values of resistance and the values of resistance of splenectomized subjects is statistically significant for both the granulocytes and mononucleates.

We found with absolute constancy that the leukocytes and, in particular, the granulocytes contained in the blood of subjects devoid of the spleen are



Statistical Analysis of Data

Polynucleates	Mononucleates
L.R.I. normal: cm² 18.540 ± σ 1.42	L.R.I normal: cm² 12.537 ± σ 1.48
L.R.I splenectomized: cm² 13.425 ± σ 2.60	L.R.I splenectomized: cm² 10.501 ± σ 2.21
Differences between average (P)* > $1\%_{10}$	Differences between average (P) > $1\%_{10}$

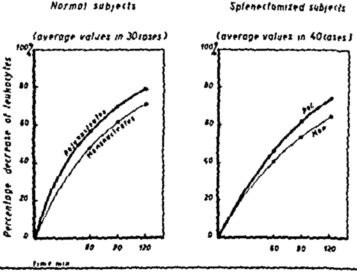
$$\text{L.R.I. (Leukocytic resistance index)} = \int_0^{\theta} y\,dx$$

FIGURE 1 Osmotic resistance of the white blood cells in peripheral blood of splenectomized subjects

more resistant than hematic leukocytes of normal subjects and that this state persists for many years after splenectomy. This finding led us to ... of a spleen- ... tome- ... uning ... much

as 20 to 30 years previously

An explanation, albeit a hypothetical one, of the above-described behavior of the resistance may be set forth on the basis of various facts we had already discovered. We have observed both in man[4] (at the operating table) and in animals[5] that the leukocytic resistances of blood contained in the splenic



Statistical Analysis of Data

Polynucleates		Mononucleates	
LRI normal	cm² 10.233 ± σ 086	LRI normal	cm² 8.633 ± σ 1.22
LRI splenectomized	cm² 8.415 ± σ 120	LRI splenectomized	cm² 7.025 ± σ 1.77
Differences between average (P)	≤1%	Differences between average (P)	≤1%

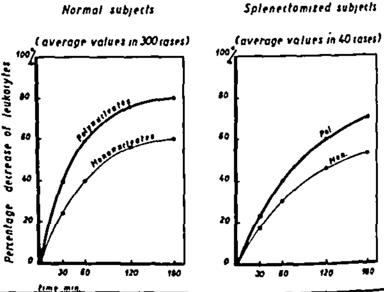
$$\text{LRI (Leukocytic resistance index)} = \int y\,dx$$

FIGURE 2 Mechanical resistance of the white blood cells in peripheral blood of splenectomized subjects

In FIGURE 3 it is clear that in man the leukocytic resistance of the blood in the splenic vein is lower than that of the splenic artery. In FIGURE 4 it is very evident that in the rabbit the leukocytic resistance of the blood in the splenic vein is lower than that of the splenic artery.

plotted The difference existing between the normal average values of resistance and the values of resistance of splenectomized subjects is statistically significant for both the granulocytes and mononucleates

We found with absolute constancy that the leukocytes and, in particular, the granulocytes contained in the blood of subjects devoid of the spleen are



Statistical Analysis of Data

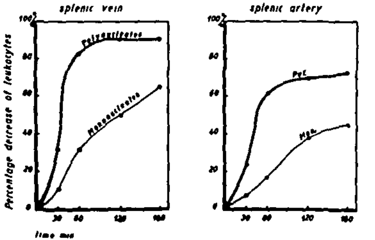
Polynucleates	Mononucleates
L.R.I normal. cm^2 18.540 ± σ 1.42	L.R.I. normal: cm^2 12.537 ± σ 1.48
L.R.I splenectomized : cm^2 13.425 ± σ 2.60	L.R.I splenectomized : cm^2 10.501 ± σ 2.21
Differences between average (P): > $1^0/_{10^8}$	Differences betwen average (P) > $1^0/_{10^8}$

L.R.I. (Leukocytic resistance index) = $\int_0^t y\,dx$

FIGURE 1 Osmotic resistance of the white blood cells in peripheral blood of splenectomized subjects

more resistant than hematic leukocytes of normal subjects and that this state persists for many years after splenectomy. This finding led us to consider the above phenomenon as a permanent biological index of a spleen-free condition Perhaps we can establish an analogy between this phenome-

In fact, during the course of our experimental and clinical research[9, 10] we have been able to demonstrate that the degree of osmotic and mechanical resistance of a given leukocytic population is proportional to the age of the elements that constitute it the resistance of leukocytes having a prevalence of morphologic-type young elements (Arneth's[11] scheme deviated to the left)



Statistical Analysis of Data	
Polynucleates	Mononucleates
L.R.I. SPLENIC VEIN · M 12921 ± σ 1.33	L.R.I. SPLENIC VEIN M 10790 ± σ 1.96
L.R.I. SPLENIC ARTERY · M 15326 ± σ 1.05	L.R.I. SPLENIC ARTERY. M 2983 ± σ 1.35
Differences between average (P) 1%	Differences between average (P) 1%

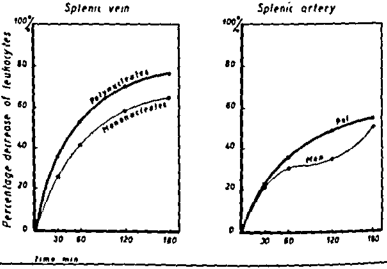
$$\text{L.R.I. (Leukocytic resistance index)} = \int_0^{t} y\,dx$$

FIGURE 4 Osmotic resistance of the white blood cells in splenic circulation of the rabbit Average of the values obtained in 15 rabbits

is much higher than that of leukocytes having a prevalence of morphological-type old elements (Arneth's scheme deviated to the right)

Since it is currently admitted that, at least in strictly physiological conditions, the younger the leukocytes the higher is their viability, it follows that there exists a direct relation between the resistance of the leukocytes and their viability

The spleen, therefore, increases the fragility of the white blood cells and this activity, at least according to the observations we have been able to make thus far, is much greater in the spleen than in other organs and tissues (liver, kidney, and peripheral tissues).[3-8] On the basis of these observations, which perhaps may now permit us to foresee the behavior of leukocytic resistances in



Statistical Analysis of Data

Polynucleates	Mononucleates
LRI SPLENIC VEIN cm^2 20409 ± σ 322	LRI SPLENIC VEIN cm^2 13986 ± σ 325
LRI SPLENIC ARTERY cm^2 12078 ± σ 435	LRI SPLENIC ARTERY cm^2 8906 ± σ 204
Differences between average (P) 1%-1‰	Differences between average (P) 1%-1‰

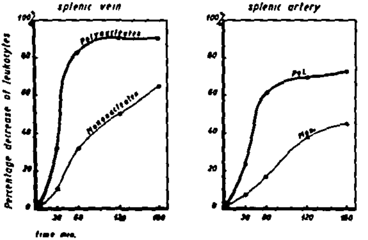
LRI (Leukocytic resistance index) = $\int_0^t y\,dx$

FIGURE 3 Osmotic resistance of the white blood cells in splenic circulation in man (on the operating table) Average of the values obtained in 10 cases

splenectomized subjects, we believe that *one may* formulate the hypothesis that the greater resistance of the white blood cells in those subjects devoid of the spleen is due to the lack of the action increasing the leukocytic fragility that the spleen has

If what has been mentioned above can be considered an interpretive hypothesis of the mechanism of the phenomenon that we have observed in [illegible] t the biological [illegible] is last question [illegible] what we have

In fact, during the course of our experimental and clinical research[9, 10] we have been able to demonstrate that the degree of osmotic and mechanical resistance of a given leukocytic population is proportional to the age of the elements that constitute it the resistance of leukocytes having a prevalence of morphologic-type young elements (Arneth's[11] scheme deviated to the left)



Statistical Analysis of Data

Polynucleates	Mononucleates
L.R.I. SPLENIC VEIN . cm² 19.921 ± σ 1.33	L.R.I. SPLENIC VEIN . cm² 10.790 ± σ 1.98
L.R.I. SPLENIC ARTERY . cm² 15.326 ± σ 1.05	L.R.I. SPLENIC ARTERY . cm² 7.983 ± σ 1.35
Differences between average (P) · 1‰	Differences between average (P) · 1‰

L R I (Leukocytic resistance index) = $\int_0^{x} y\,dx$

FIGURE 4 Osmotic resistance of the white blood cells in splenic circulation of the rabbit Average of the values obtained in 15 rabbits

is much higher than that of leukocytes having a prevalence of morphological-type old elements (Arneth's scheme deviated to the right)

Since it is currently admitted that, at least in strictly physiological conditions, the younger the leukocytes the higher is their viability, it follows that there exists a direct relation between the resistance of the leukocytes and their viability

Therefore it appears, in concluding our present series of studies, that we can formulate the hypothesis that the white blood cells of a splenectomized subject have a greater viability than those of a normal subject and that the

spleen, therefore, intervenes also in perfectly normal conditions in the complex mechanism of regulating the viability of the white blood cells.

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DYNAMICS OF HEMOPOIETIC PROLIFERATION IN MAN AND MICE STUDIED BY H³-THYMIDINE INCORPORATION INTO DNA*

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Tissues in the adult can be divided into two categories, one of which is being replaced continually and one in which the cell populations remain essentially stable throughout adult life [1] The possible relationship between neoplasia and the rate of cellular turnover in tissues has not been explored satisfactorily, although this problem, particularly with neoplasia of the hematopoietic tissue, has received considerable attention To Virchow, leukemia was a result of an increased and uncontrolled proliferation of cells resulting in local invasion and distant metastasis [2] Ultimately the tumor cells became a parasitic burden to the host, and death ensued There can be no doubt that in some respects Virchow's concept is essentially correct However Bierman[3] has felt that certain types of leukemia might well result from a defect in the removal system, and he proposed a balance hypothesis for neoplasia, particularly the leukemias The resolution of these differences becomes of great importance because, on the one hand, therapy might well be aimed at the control of new cell proliferation and, on the other hand, at correcting a faulty removal mechanism

In order to understand better the mechanisms of neoplasia, an understanding of the processes of normal cell proliferation and removal is essential A great amount of effort has been devoted to the study, by various techniques and in various species, of the characteristics of proliferation of the different hematopoietic cell systems from the stem forms to the mature elements Classic techniques used have included marrow suppression by radiation or chemical compounds,[4, 5] mitotic counts and cell enumeration in lymph nodes,[6] cross-circulation experiments,[7, 8] parabiosis,[8, 9] transfusions,[10, 11] arteriovenous catheterization and cross circulation,[12] cell and nucleus volume measurements,[13, 14] cannulation of the lymphatic ducts,[15, 16] cell labeling with dyes,[17, 19] *in vitro* cell culture,[20-23] and leukocyte depletion techniques [24, 25] Labeling with radioactive or stable isotopes combined with the classic techniques resulted in significant progress in this area Outstanding is the classic work in which the life span of human erythrocytes was determined to be 120 days [26] Other isotopic methods employed to study the several hematopoietic cell systems include the tagging[27, 31] of desoxyribonucleic acid (DNA)

*The work reported in this paper was supported by the United States Atomic Energy Commission, Washington, D C

with P^{32} or[32,33] C^{14}, S^{35} labeling of cystine,[36] Fe^{59} labeling of leukocyte cytochrome oxidase[37] and of hemoglobin,[38] and Cr^{51} labeling of erythrocytes.[39] These and other data have been used in attempts to construct models of the dynamics of hematopoiesis.[1,3,5-8,12-14,16-22,34,40,41] With the exception of studies of the life span of the erythrocyte, these approaches have led to conflicting results because of inherent disadvantages and uncertainties, particularly in the analysis of data acquired.

Reichard and Estborn[42] and Friedkin[43] have demonstrated that thymidine is efficiently utilized in the formation of DNA by growing cells. The recent development of tritiated thymidine in this laboratory by Hughes[44,45] has made the selective *in vivo* tagging of DNA practicable in proliferating cellular tissues of mammals. Thymidine is a specific precursor for DNA. The beta particle emitted by tritium is of low energy (0.019 mev) and permits high-resolution autoradiography. Thus, DNA synthesis and the dynamics of cell proliferation can be followed in specific cell lineages. The kinetics of cell turnover can be followed by grain counts over specific cell types in contrast to older techniques that usually involved either studies on mixed cell populations or complicated cell separation procedures. Because of the preceding desirable characteristics, studies with tritiated thymidine were initiated to study the proliferative activity of tissues in man and animals.

Previous studies in this laboratory[46] have indicated that intravenous H^3-thymidine disappears from the blood stream in a matter of minutes (availability time short). It is incorporated in part into DNA and in part, is catabolized to water and to other nonvolatile compounds. Because of the above-described desirable characteristics, studies with tritiated thymidine were initiated to study the proliferative activity of tissues in man and animals.

Some studies using *in vitro* techniques were made with patients with hemopoietic diseases and with normal individuals. Studies *in vivo* with animals had shown that satisfactory labeling of cell renewal systems was feasible without any apparent radiation effects. Computations showed that the total body dose from tritium would be acceptable after even larger doses of material were administered intravenously. Admittedly, the long-term somatic and possible genetic effects have not been evaluated, hence its use should be limited for the time being to adults past procreative age with short life expectancies.

Materials and Methods

Studies on man. To date, two adult male patients with diagnoses of brain tumor, but otherwise in good condition and without evidence of hemopoietic or nutritional dysfunction, have received intravenous H^3-thymidine. The first patient was decerebrate, the life expectancy of each patient was limited. The first patient received 4 injections, approximately 1 month apart, of 9 to 19 mc. tritiated thymidine with high specific activity. The second patient similarly received 8.3 mc. Following each administration serial venous blood and bone marrow preparations were made. Clinical and biochemical details will be published in detail elsewhere.[46]

Blood leukocyte preparations were made by sedimenting red cells with

dextran and then concentrating the leukocytes by centrifugation [47] Leukocytes were resuspended in plasma to give a concentration of 100,000/cu mm, and smears were made Bone marrow aspiration was performed from either the iliac crests or the sternum, using a Turkel needle Approximately 1 0 ml of the marrow was withdrawn into each of two syringes for preparation of particle smears which were rapidly dried Some marrow was allowed to clot for sections Squash preparations[48] were made for study of mitotic figures Autoradiographs were made, using stripping film,[49] and Wright's stain technique (careful *p*H control) was applied after suitable exposure and development [50] Squash preparations were stained by a modification of Feulgen's method [48]

In vitro studies were made with venous blood and bone marrow by incubating for 1 hour with H^3-thymidine (2 μc ml) * Dextran sedimentation was used with peripheral blood for concentrating leukocytes, and autoradiographs were prepared Smears were then made To date, the subjects include 2 patients with multiple myeloma, 1 with chronic myelogenous leukemia in acute myeloblastic crisis, 1 with chronic lymphatic leukemia, 1 with agnogenic myeloid metaplasia, 2 with infectious mononucleosis, and 3 with polycythemia vera The subjects for comparison of *in vitro* uptake into blood and bone marrow cells were 6 healthy male laboratory employees between 28 and 44 years of age

Mice were given a single intraperitoneal injection of H^3-thymidine, and were sacrificed serially Specimens of blood, bone marrow, and tissues were prepared for autoradiographs with stripping film, as described below

In tabulating cells of the marrow or blood a cytological identification was made and grains overlying the cell nuclei were counted For determination of the percentage of cells labeled, a cell was recorded as labeled if there were more than three grains As work progressed it became evident that this method resulted in an underestimation of the number of labeled cells and that it would be feasible to subtract the random background count by appropriate statistical means These computations are not included in this report, nor is the statistical analysis of the variation in grain count over specific cell types with time after injection These data will be reported separately

In this paper emphasis will be given to studies that bear on the kinetics of hematopoiesis in man The kinetic analysis will be published later

Results

Results of the *in vitro* studies in man are shown in FIGURES 1 to 4 and in FIGURE 6 The rapidity of plasma clearance and appearance of plasma-tritiated water is shown in FIGURE 1 Isotopic equilibrium is approached within one hour Complete metabolic details will be published elsewhere.[41] Intense labeling of primitive proliferating cells was seen at 15 min (FIGURE 2 1) The term primitive proliferating pool of cells (PPP) is used as an interim solution to semantic difficulties In this category are included cells that have previously been called hemohistioblasts, hemocytoblasts, stroma cells, stem cells, primitive mesenchymal cells, reticulum cells, and histiocytes.

* Subsequently it has been shown that 0 5 μc /ml is adequate

A new general term is used because there is no universal agreement on terminology. This specific term is used because, as will be discussed, these cells are labeled promptly and intensely and, in all probability, represent a diffuse primitive mesenchymal pool. At the time interval of 15 min. the primitive proliferating cells always showed the most intense label. At 30 min., 60 per cent of these cells were labeled heavily. Of these labeled cells, approximately 60 per cent had more than 40 grains, 37 per cent, more than 75 grains, and 30 per cent, more than 100 grains. By 5 days the percentage of these

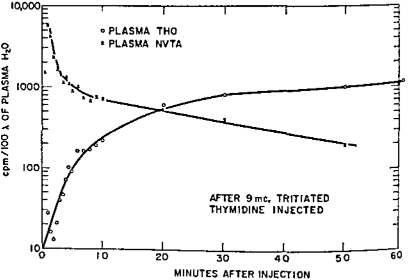
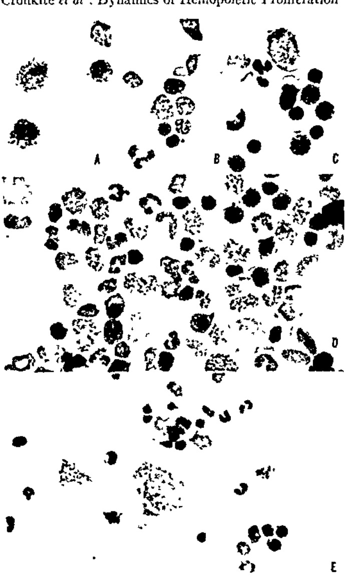


FIGURE 1. Curves for appearance of tritiated water (THO) and disappearance of nonvolatile tritium activity (NVTA) from the plasma of a human being after intravenous injection of tritiated thymidine. The NVTA in the early minutes is H^3-thymidine. At later intervals the NVTA may consist of H^3-thymidine and metabolic products of thymidine such as β-aminoisobutyric acid.

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that the act of making smears destroys many of the PPP cells and that, ordinarily, these cells would be called degenerating cells, but that the presence of label proves they were actively synthesizing DNA and are not senescent degenerating cells.

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necessity of the marrow to dispose of an average of 2×10^{11} normoblastic nuclei per day, no direct evidence has been found to date for the mechanism or for the site of removal of these nuclei by this labeling technique. Although data are incomplete, it appears that the percentage of labeling reached a maximum in the entire erythrocytic series between 12 and 24 hours after injection In the curves for the grain counts and percentage of labeling for different stages of maturity, no visible label was seen after 5 days in the erythroid series

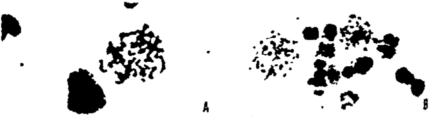


FIGURE 3 Demonstration of mitoses in human bone marrow by Feulgen's squash technique (A) Mitoses in normal marrow, no H^3-thymidine (B) Labeled and unlabeled mitosis 24 hours after 19 mc of H^3-thymidine intravenously



FIGURE 4 Morphologic appearance of labeled circulating blood cells after 9 mc of H^3-thymidine intravenously (A and B) Mononuclear cells. (C) Band neutrophilic granulocyte (D) Segmented neutrophilic granulocyte

Distinct myelocytic labeling was shown one hour after injection (FIGURE 2C). Labeling of the entire myeloid series attained a maximum between the third and fourth days Labeled band forms were seen first in the marrow on the third day Labeling was still evident through the fifth day. By

[illegible] le

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[illegible] us time intervals In FIGURE 3A is an example from a Feulgen squash preparation of mitosis in human marrow in an individual who had not been given H^3-thymidine An

example of a labeled and non-labeled mitosis is illustrated in FIGURE 3B, at 24 hours after the injection of H^3-thymidine. At 2 hours after injection, 23 of 25 mitotic figures were labeled.

It is significant that the highest grain counts were in the PPP cells and that the grain counts of differentiating erythroid and myeloid cells appear to

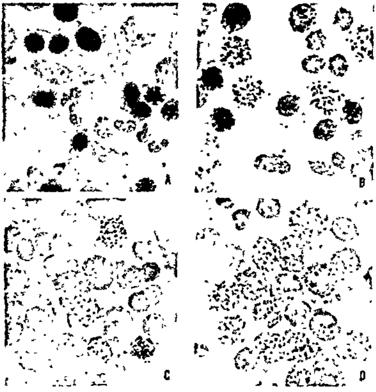


FIGURE 5. *In vitro* labeling of hemopoietic elements. (*A*) Unlabeled comparison marrow for background. (*B*) Same marrow incubated 60 min. with 2 μc. H^3-thymidine per ml. of marrow. (*C*) Marrow of multiple myeloma. Note labeled normal elements and no labeling of myeloma cells. (*D*) Labeling of circulating leukemic cells from patient with acute myeloblastic crisis.

decrease successively with time by factors of two, as does the grain count in the PPP. As the grain count decreases, the percentage of labeled cells in the erythroid myeloid and megakaryocytic series increases. More work is necessary to confirm these trends conclusively.

The appearance of labeled cells in the peripheral blood is shown in FIGURE 6. Mononuclear cells include monocytes, as well as large and medium (not small) lymphocytes. Labeled mononuclear cells were seen during the first 24 hours,

and the maximum percentage of labeled cells appeared at 2½ days. The percentage of visibly labeled mononuclears decreased slowly. None was seen after the tenth day. It is to be noted that this is a composite curve and that there is good reason to believe that the curve for the various types of mononuclear cells will be different. These cells are illustrated in FIGURES 4A and 4B. Labeled small lymphocytes were not seen on the first day. Labeled small lymphocytes were seen very rarely in the peripheral blood between the second and tenth days. The percentage labeled was certainly much less than 0.1 per cent.

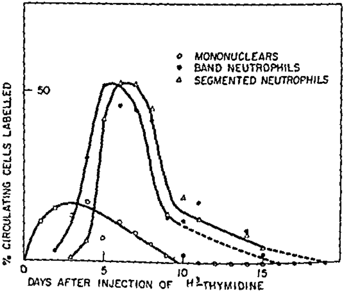


FIGURE 6. Curves for appearance and disappearance of visible labeling of circulating leukocytes.

Band neutrophils (FIGURE 4C) were absent on day 1. A small percentage was seen on day 2, the percentage of labeled cells climbed sharply to 55 per cent on day 5. Labeled segmented cells (FIGURE 4D) reached the maximum percentage 1 day later than did the band forms. Segmented neutrophils decreased, as illustrated (FIGURE 6). Very few visibly labeled bands or segmented cells (a fraction of 1 per cent) were found after the fifteenth day. Labeled eosinophils, although too few in number to quantitate, were found

esulted in labeling of all cell
unlabeled normal marrow for
an estimate of background grain is compared to FIGURE 5B, in which the

studies from this laboratory indicated that less than 0.1 per cent of all cells were labeled.[51]

In vitro uptake of thymidine was minimal in the abnormal cells of both chronic lymphatic leukemia and multiple myeloma, however, uptake was marked in normal-appearing islands of hematopoiesis (FIGURE 5C). In contrast to the very sluggish or absent DNA synthesis by chronic lymphatic leukemia cells and marrow myeloma cells there was very active DNA synthesis by circulating leukemic cells from a case of myelogenous leukemia in a terminal acute myeloblastic crisis (FIGURE 5D). Detailed results of *in vitro* studies of blood and bone marrow in disease will be reported later.[52]

The studies on mice will be summarized very briefly. At 15 min after injection a very heavy and widespread labeling of PPP cells was found throughout lymph nodes, spleen, bone marrow, and loose connective tissue of intestinal villi, submucosae, and areolar tissue. At early time intervals labeling of small lymphocytes was negligible throughout the body. Light labeling was found at later time intervals. The sequence of appearance and disappearance of labeled cell types in the blood is similar to man in respect to trends, but somewhat shorter in time, therefore graphic presentation will not be made. Results in mice are similar to those obtained by Bryant and Kelly.[33]

Thoracic duct cannulation in the dog followed by injection of the labeled thymidine has demonstrated that large labeled cells are present as early as 10 min after injection. These cells have a basophilic cytoplasm and large immature nuclei, occasionally with a nucleolus. In time the percentage of the large labeled cells decreases and an increasing number of small to medium-sized cells that have an intensely blue cytoplasm are seen. The nuclei of these cells are not heavily clumped, as in the typical small lymphocyte, although some of these cells approach 7 μ in diameter. Typical small lymphocytes with labels were not seen.

Again, the widespread and unexpected observation of intense and rapid DNA synthesis in primitive proliferating cells in hemopoietic and connective tissues throughout the body suggests that large numbers of mesenchymal cells undergo steady and rapid turnover throughout the body. Whether these cells have similar capacities and are totipotential or whether they represent a family of mesenchymal cells is not clear.

Discussion

The present data were obtained on human beings with undisturbed hematopoiesis. The techniques used made it possible to study the behavior of individual cells and cell lineages. Therefore the data obtained should allow characterization of the different cell systems in the hematopoietic tissues.

A discussion of the general picture of steady-state cell proliferation in tissues that are being replaced continually involves the following. The mass of an organ or tissue normally is essentially constant. The turnover rate or number of cells produced per unit time varies with turnover time and population size. Turnover time depends upon the average life span of the individual cells. Study of turnover time and rate is dependent upon knowledge

of size of the total population and its compartmentation; the latter may be both anatomical and functional. Upon completion of one or a series of multiplicative mitoses, differentiation into mature functioning elements occurs. These cellular systems can be divided into three basic compartments involving [illegible]
multiple [illegible]
ary may [illegible]
cycle. However, the ultimate flow rate from the total generative and maturative compartments must equal the sum of death and utilization rates to maintain a relatively constant mass.

Much confusion has arisen from failure to define carefully the life span or cell survival time. The interval between successive mitoses is the generation time. The latter is subdivided into mitotic time and intermitotic time. The latter is again subdivided into resting periods before and after DNA synthesis (DNA doubling time). In general, for a cell line it is probable that these times are variable and distributed around constant means for each subdivision of the generative compartment. Cells from the generative maturation compartments flow into the functioning compartment that can be divided into use, storage, transit, and death. It must be specified carefully whether life span refers to total life span from the earliest cell in the generative compartment to death or whether life span refers to time in a specific compartment, for example, time in circulating blood, time in storage, or time in tissues. Various cellular proliferative schemes and mathematical models have been proposed. Osgood *et al.*,[20,21,22] Lajtha *et al.*,[23] Weicker,[13,14] Patt,[40] Hamilton,[42,43] and Cronkite[41] have developed and published models. It would be premature from the present data to present new, or to change drastically, the existing concepts. In collaboration with us, von Foerster[37] is developing a generalized mathematical concept of proliferating steady- and nonsteady-state cell populations. The following, however, can be concluded from the present data.

First, it must be emphasized that certain basic assumptions have been made with respect to thymidine and DNA. These basic assumptions are (1) the tritium label on the thymidine is stable and does not exchange with available hydrogen, (2) when incorporated into DNA, thymidine is stable [illegible] (3) [illegible]

significantly reutilized in new DNA synthesis. The latter assumption has been challenged by Hamilton,[42-44] who has evidence that indicates either that DNA of lymphocytes is reutilized or that there is a long-lived population of lymphocytes [illegible]

The significance of p[illegible] cell, [illegible]
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DNA synthesis time to generation time of this generative cycle. If the [illegible] division [illegible]
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the label only by factors of two Actual grain counts ... vidual cells are dependent upon ... it is assumed that the geor ... is constant although, adn ... grain count is dependent ... radioactivity in the cell If the rate of synthesis of DNA is constant for all similar cells and if the cell was synthesizing DNA throughout the entire availability period each ... would have essentially the same radioactivity ... cells that com... became av... per unit ti... will follow the Poisson distribution ... of the preceding will result in a moderately skewed Poisson distribution for grain count over cells It is believed that quantitative studies on cellular behavior can be performed using the mean grain count

The present data tend to support the concepts of Ottesen[28] and Hamilton[32,33] that there are two populations of lymphocytes one rel... lived and the other long-lived ... lymph... ... to third day and there... ... lymph nodes and the spleen of the mouse suggest that the larger lymphocytes are precursors of the smaller, and that the small lymphocytes are nondividing progeny of the former, since intense label is seen in the large cells at 15 minutes after injection, whereas small lymphocytes are not labeled until later These observations are consistent with Trowell[34] who considers the small lymphocyte a nondividing ... with the recent scheme ...

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ap... th... fro... to a ... mitoses of precursor ... tion of labeled mate...

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curve The curve of FIGURE 6 obtained by autoradiographs of cytologically identified cells is similar to the curves that Ottesen,[35] Hamilton,[22,44] and Resegotti[37] derived from specific activity data on $Na_2HP^{32}O_4$ labeling of DNA, C^{14}-adenine-guanine labeling of DNA, and Fe^{59} labeling of hemin in cytochrome oxidase, respectively. It appears that the foregoing individuals did not incorporate into their models the exponential decrease in intensity of label by mitosis Accordingly, it is open to question whether the mathematical analyses used to date are adequate for extracting information on total life span or time spent in the blood. An extensive mathematical analysis of the general problem is in preparation.[55]

The data on percentage labeling of mitotic figures in the marrow are not complete However, it is quite evident that the picture will be complex since one is dealing with a mixture of proliferating cells: PPP, erythroid, granulocytic, and megakaryocytic However, it appears clear that the percentage of labeled mitotic figures of any cell type will increase to approximately 100 per cent after a time interval equal to the post-DNA synthesis period plus 1 mitotic time. Following this there should be a plateau that approximates DNA synthesis time. As cells that were in the pre-DNA synthesis period enter mitosis, the percentage of labeling will decrease and oscillate until it settles down to a constant value that will represent the ratio of DNA synthesis time to generation time. Of course, the intensity of the label will decrease by a factor of 2 with each mitosis In fact, such a sequence of events has been observed by Quastler *et al.*[56] in the single-cell population of the generative part of the small intestine.

In view of the foregoing discussion, the increase in percentage of labeled cells in the marrow with time is disturbing. This implies that there is either a storage pool of labeled DNA precursor (thymidine or thymidylic acid) that has not been appreciated or a marked diminution in ratio of DNA synthesis time to total generation time with successive divisions. In the latter case a smaller percentage of cells at, for example, the myelocyte level would be labeled than at the more immature levels. As the immature PPP-labeled cells divide, the percentage of labeled myelocytes would increase to the ratio of DNA synthesis time to generation time for the most immature member of the series The time involved to attain the maximum percentage of labeling and the diminution in the label should be, in this case—given the number of divisions involved and the quotient of time by the number of divisions—the average generation time for the series.

Our studies on grain count in various cell lineages with maturation are not complete However, it is clear that if there are doubling divisions of erythroblasts or myelocytes, none of the mature forms can have more than

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work Weicker[13,14] indicates that there are 1 arithmetic and 3 doubling divisions between the proerythroblast and orthochromatic normoblast The observations of Yoffey[16] on the progressive absolute increase in the number of cells of increasing maturity in the marrow of the guinea pig suggest that there is 1 arithmetic division followed by 3 doubling divisions between the myeloblast and the band granulocyte In the erythroid series there is 1 arithmetic and 4 doubling divisions between the proerythroblast and the orthochromatic normoblast If one assumes that all identifiable cell types go through doubling divisions and that there is a common primitive proliferating pool of cells that divides arithmetically, feeding cells into each multiplication compartment, all divisions in both the myeloid and erythroid series would

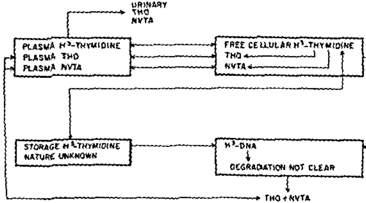


FIGURE 7 Schematic presentation of the metabolism of H³ thymidine and route of incorporation into DNA

be doubling The latter view is suggested by our labeling techniques that show intense labeling of the PPP cells More quantitative absolute cell counts on the marrow contents combined with labeling techniques are essential to answer the problems raised by and discussed in this work

Since these are observations that can be resolved only by supposition about availability time of the labeled material or storage of a metabolic product that can go to DNA, the following scheme of the metabolic pathways of the labeled thymidine has been proposed as a working hypothesis (FIGURE 7) It is visualized that thymidine rapidly diffuses into tissues after intravenous injection and reaches isotopic equilibrium The material then has certain alternatives It can be catabolyzed to water, to nonvolatile tritium compounds, stored as an intermediate or protected thymidine for future DNA synthesis, or may be synthesized immediately into DNA The relative importance and sizes of these compartments are not known It has been shown by Rubini *et al* [16] that about one third of the injected dose is recovered as tritiated water (THO) and less than one sixth as nonvolatile tritium activity (NVTA), in the urine More than 50 per cent of the material is unac-

counted for, or has actually been incorporated into DNA. Although the existence or absence of an active transport mechanism for thymidine is not known, it is clear that the passage into cells and the incorporation into DNA are rapid. Without evoking a special mechanism, we visualize that cells in DNA synthesis act effectively as sinks for H^3-thymidine, thus maintaining a maximal diffusion gradient between intra- and extranuclear thymidine while labeled thymidine is available. In fact, in one of our patients who weighed 60 kg. it can be shown that H^3-thymidine would have to have been concentrated in order to obtain the autoradiographs. A rough approximation of the number of cells in the body can be made, assuming the body to be 50 per cent cells and that the red cell is an average-sized cell, then $60 \times 10^3 \times 5 \times 10^6 = 3.0 \times 10^{14}$ cells in the body. This number could be *in error by a factor of 10.* *If H^3-thymidine were distributed* equally to all cells to be catabolyzed or incorporated into DNA, the maximum number of disintegrations per unit time possible over any cell after a 9 mc. dose could be calculated easily as follows:

$$9 \times 2.22 \times 10^9 = 19.98 \times 10^9 \text{ dpm injected, or}$$

$$\frac{19.98 \times 10^9 \times 4.3 \times 10^4}{3.0 \times 10^{14}} = 2.8 \text{ d/month}$$

This represents 2.8 disintegrations per cell per month on the average. Grain counts over cells varied from 1 to 100 grains. With a constant amount of radioactivity per cell, one would expect the disintegrations to vary with the Poisson distribution. If one assumed a radioautographic efficiency of 10 per cent, it would be *necessary to have 10 times the number of disintegrations* to get the observed grain counts, or a range of 10 to 1000 d/month. Uniform distribution allows on an average only 2.8 d/month, hence a concentration mechanism by cells synthesizing DNA certainly exists.

The existence of a large mass of primitive mesenchymal tissue throughout ... not new. Histolo- ... has been responsi- ... t to monophyletic ... he intense cellular activity of these cells in the normal adult man and in the normal mouse was unexpected. On a quantitative basis, since these cells are present throughout the body, the turnover rate may equal or even exceed the turnover rates of the epithelial tissues. This is a subject for quantification. These data *showing high cellular DNA synthesizing activity* and *dilution of the* label, ... the mono- ... n some ... f being ... ls of all ... ificance ... asis has always been considered to be only a morbid process. However, the presence of circulating primitive proliferating cells destined to divide[44] suggests that

this is a normal process providing a mobile pool of possible totipotential mesenchymal cells for defense and repair throughout the body, in addition to providing a mechanism for reseeding atrophic bone marrow from normal marrow

One might speculate about the potential uses of this labeling agent for DNA. Its uses in studies as reported herein and for general problems of kinetics of cell proliferation are obvious. However, there is a series of problems in which the agent might be useful. In the assessment of potential anticancer drugs one is interested in the site of action. Are the drugs antimitotic? Anti-DNA synthesis? Anticellular? Anti-DNA synthesis activity could be evaluated precisely by the use of H^3-thymidine in normal and malignant tissues *in vivo* and *in vitro*. The role of endocrine factors (such as cortisone and estrogens) on cell proliferation could be evaluated. It has long been known that preirradiation treatment of animals with estrogens will increase resistance of the animals and that postirradiation treatment with cortisone will increase mortality. The mechanism of action of these two effects might well be solved by a study of the effect of the treatments on the state of the PPP cells.

Some of the present data suggest that in acute leukemia there is a real increase in cell proliferation of the leukemic cells, whereas evidence for extraproliferation in the chronic leukemias and multiple myeloma is lacking to date; in fact, there appears to be less labeling than in the residual normal tissue. It is tempting to suggest that this indicates a longer life span; however, the data will not prove this point. A fraction of 1 per cent increase in production or a fraction of an increase in life span, neither of which could be detected by present techniques, would give the same results of increasing masses of lymphocytes or plasmacytoma cells. However, the labeling technique will identify the numbers of the neoplastic group that are proliferating; it thus introduces another method for classification of neoplastic tissue by function, as well as by appearance.

Summary and Conclusions

Tritiated thymidine has been used in animals and in human beings with normal and abnormal hematopoiesis to study the kinetics of bone-marrow cell proliferation. Thymidine is taken up by the nucleus only during DNA synthesis preparatory to cell division. Its intracellular location was determined and quantified by means of stripping film autoradiography and classic cytology.

In the bone marrow of human beings with normal hematopoiesis given H^3-thymidine intravenously, intense labeling of primitive proliferating cells was observed within minutes. Maximal labeling of erythrocyte precursors occurred at 12 to 24 hours, myeloid precursors, between the third and fourth days. Maximal labeling of megakaryocytes was observed on the fifth day. Labeling in the peripheral blood of large mononuclear cells occurred early; the peak labeling of granulocytes occurred on the fifth and sixth days. H^3-thymidine incubated with normal human bone marrow resulted in heavy labeling in all cell series; normal venous blood showed labeling of only a small

percentage of the large mononuclear cells. The pattern of H³-thymidine uptake with *in vitro* incubation of bone marrow and venous blood from patients varied with different hematopoietic disorders. A preliminary analysis of the quantitative results in terms of the kinetics of hematopoietic cell proliferation in health and in disease is made, and the requirements for resolution of the complex problems involved are indicated.

The intense labeling of the fragile group of primitive mesenchymal cells in the lymph nodes and bone marrow and their appearance in lymph and blood in small numbers prove that immature cells capable of division are continually migrating throughout the body. It is believed that this is a new observation, the significance of which is not yet clear. However, it is entertaining to consider this phenomenon as another general biological protective measure to make totipotential cells available at any time or place for defense, repair, or repopulation of damaged bone marrow, as in radiation or other total or partial aplasias of the marrow.

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